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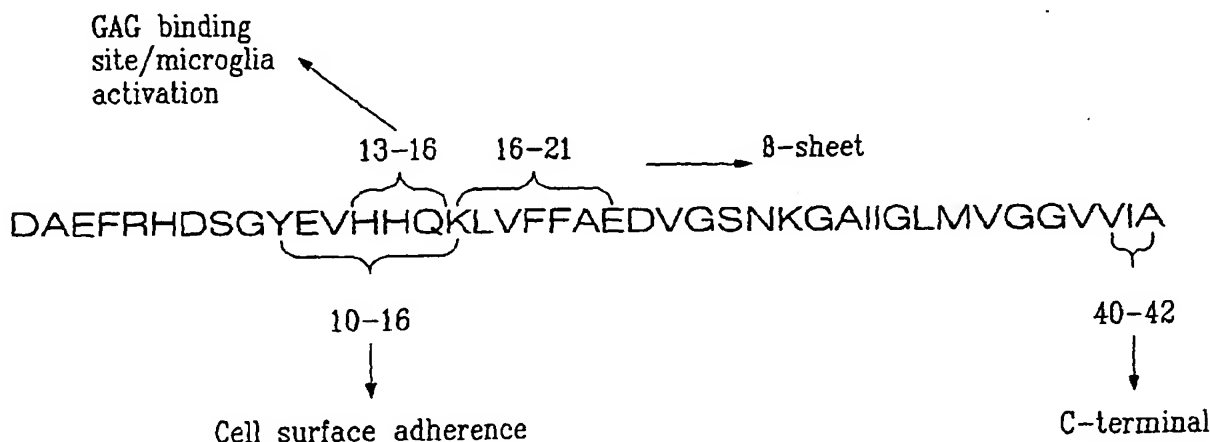
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(54) Title: **VACCINE FOR THE PREVENTION AND TREATMENT OF ALZHEIMER'S AND AMYLOID RELATED DISEASES**



(57) Abstract: The present invention relates to a stereochemically based "non-self" antigen vaccine for the prevention and/or treatment of Alzheimer's and other amyloid related diseases. The present invention provides a vaccine for the prevention and treatment of Alzheimer's and other amyloid related diseases, which overcomes the drawbacks associated with using naturally occurring peptides, proteins or immunogens.

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# VACCINE FOR THE PREVENTION AND TREATMENT OF ALZHEIMER'S AND AMYLOID RELATED DISEASES

## BACKGROUND OF THE INVENTION

5           The present invention relates to a new stereochemically based "non-self" antigen vaccine for the prevention and/or treatment of Alzheimer's and other amyloid related diseases.

          Amyloidosis refers to a pathological condition characterized by the presence of amyloid fibers. Amyloid is a generic term referring to a group of diverse but  
10       specific protein deposits (intracellular and/or extracellular) which are seen in a number of different diseases. Though diverse in their occurrence, all amyloid deposits have common morphologic properties, stain with specific dyes (*e.g.*, Congo red), and have a characteristic red-green birefringent appearance in polarized light after staining. They also share common ultrastructural features and common x-ray  
15       diffraction and infrared spectra.

          Amyloid-related diseases can either be restricted to one organ or spread to several organs. The first instance is referred to as "localized amyloidosis" while the second is referred to as "systemic amyloidosis".

          Some amyloidotic diseases can be idiopathic, but most of these diseases  
20       appear as a complication of a previously existing disorder. For example, primary amyloidosis can appear without any other pathology or can follow plasma cell dyscrasia or multiple myeloma. Secondary amyloidosis is usually seen associated with chronic infection (such as tuberculosis) or chronic inflammation (such as rheumatoid arthritis). A familial form of secondary amyloidosis is also seen in  
25       Familial Mediterranean Fever (FMF). This familial type of amyloidosis, as one of the other types of familial amyloidosis, is genetically inherited and is found in

specific population groups. In these two types of amyloidosis, deposits are found in several organs and are thus considered systemic amyloid diseases. Another type of systemic amyloidosis is found in long-term hemodialysis patients. In each of these cases, a different amyloidogenic protein is involved in amyloid deposition.

- 5           “Localized amyloidoses” are those that tend to involve a single organ system. Different amyloids are also characterized by the type of protein present in the deposit. For example, neurodegenerative diseases such as scrapie, bovine spongiform encephalitis, Creutzfeldt-Jakob disease and the like are characterized by the appearance and accumulation of a protease-resistant form of a prion protein
- 10 (referred to as A $\beta$ Sc or PrP-27) in the central nervous system. Similarly, Alzheimer's disease, another neurodegenerative disorder, is characterized by neuritic plaques and neurofibrillary tangles. In this case, the plaque and blood vessel amyloid is formed by the deposition of fibrillar A $\beta$  amyloid protein. Other diseases such as adult-onset diabetes (Type II diabetes) are characterized by the localized accumulation of
- 15 amyloid in the pancreas.

Once these amyloids have formed, there is no known, widely accepted therapy or treatment which significantly dissolves the deposits *in situ*.

- Each amyloidogenic protein has the ability to organize into  $\beta$ -sheets and to form insoluble fibrils which get deposited extracellularly or intracellularly. Each
- 20 amyloidogenic protein, although different in amino acid sequence, has the same property of forming fibrils and binding to other elements such as proteoglycan, amyloid P and complement component. Moreover, each amyloidogenic protein has amino acid sequences which, although different, will show similarities such as regions with the ability to bind to the glycosaminoglycan (GAG) portion of
- 25 proteoglycan (referred to as the GAG binding site) as well as other regions which will promote  $\beta$ -sheet formation.

In specific cases, amyloidotic fibrils, once deposited, can become toxic to the surrounding cells. As per example, the A $\beta$  fibrils organized as senile plaques have been shown to be associated with dead neuronal cells and microgliosis in patients with Alzheimer's disease. When tested *in vitro*, A $\beta$  peptide was shown to be capable  
5 of triggering an activation process of microglia (brain macrophages), which would explain the presence of microgliosis and brain inflammation found in the brain of patients with Alzheimer's disease.

In another type of amyloidosis seen in patients with Type II diabetes, the amyloidogenic protein IAPP has been shown to induce  $\beta$ -islet cell toxicity *in vitro*.  
10 Hence, appearance of IAPP fibrils in the pancreas of Type II diabetic patients could contribute to the loss of the  $\beta$  islet cells (Langerhans) and organ dysfunction.

People suffering from Alzheimer's disease develop a progressive dementia in adulthood, accompanied by three main structural changes in the brain: diffuse loss of neurons in multiple parts of the brain; accumulation of intracellular protein deposits  
15 termed neurofibrillary tangles; and accumulation of extracellular protein deposits termed amyloid or senile plaques, surrounded by misshapen nerve terminals (dystrophic neurites). A main constituent of these amyloid plaques is the amyloid- $\beta$  peptide (A $\beta$ ), a 40-42 amino-acid protein that is produced through cleavage of the  $\beta$ -amyloid precursor protein (APP). Although symptomatic treatments exist for  
20 Alzheimer's disease, this disease cannot be prevented nor cured at this time.

The use of a vaccine to treat Alzheimer's disease is possible in principle (Schenk, D. *et al.*, (1999) *Nature* 400, 173-177). Schenk *et al.* show that, in a transgenic mouse model of brain amyloidosis (as seen in Alzheimer's disease), immunization with A $\beta$  peptide inhibits the formation of amyloid plaques and the  
25 associated dystrophic neurites. In that study, a vaccine using the human aggregated all-L peptide as immunogen prevented the formation of  $\beta$ -amyloid plaque, astrogliosis and neuritic dystrophy in vaccinated transgenic mice.



However, it is apparent that there are a number of drawbacks to using an endogenous protein as a vaccine (or a protein naturally present in the animal being vaccinated). Some of these drawbacks include:

- 5                   • Possible development of autoimmune disease due to the generation of antibodies against "self" protein.
- Difficulty in eliciting an immune response due to the failure of the host immune system to break tolerance.
- Possible development of an acute inflammatory response in the brain due to antibody-mediated phagocytosis by microglia cells.
- 10               • Development of anti-idiotypic antibodies.

#### SUMMARY OF THE INVENTION

The present invention relates to a stereochemically based "non-self" antigen vaccine for the prevention and/or treatment of Alzheimer's and other amyloid related diseases. One aim of the present invention is to provide a vaccine for the prevention  
15 and treatment of Alzheimer's and other amyloid related diseases, which overcomes the drawbacks associated with using naturally occurring peptides, proteins or immunogens, such as a fibril protein (e.g., beta Amyloid).

The term "fibril and/or amyloid peptide" as used herein, encompasses both monomeric, oligomeric and soluble  $\beta$ -amyloid or any fibril protein, as discussed  
20 herein, as well as any other structural variants that may occur naturally, are synthetically constructed or correspond to a known fibril protein. Specifically, a fibril and/or amyloid peptide consists of at least 3 amino acids from a fibril peptide, such as amyloid or any structural variant thereof. The term "corresponds to" is used herein to mean that a polynucleotide sequence is homologous (i.e., is identical,  
25 not strictly evolutionarily related) to all or a portion of a reference polynucleotide sequence, or that a polypeptide sequence is identical to a reference polypeptide

sequence. In contradistinction, the term "complementary to" is used herein to mean that the complementary sequence is homologous to all or a portion of a reference polynucleotide sequence. For illustration, the nucleotide sequence "TATAC" corresponds to a reference sequence "TATAC" and is complementary to a reference  
5 sequence "GTATA".

The term "amyloid related diseases" includes diseases associated with the accumulation of amyloid either in soluble or insoluble (plaque) forms, which can either be restricted to one organ, "localized amyloidosis", or spread to several organs, "systemic amyloidosis". Secondary amyloidosis may be associated with  
10 chronic infection (such as tuberculosis) or chronic inflammation (such as rheumatoid arthritis), including a familial form of secondary amyloidosis which is also seen in Familial Mediterranean Fever (FMF). Another type of systemic amyloidosis found in long-term hemodialysis patients. Localized forms of amyloidosis include, without limitation, diabetes type II and any related disorders thereof, neurodegenerative  
15 diseases such as scrapie, bovine spongiform encephalitis, Creutzfeldt-Jakob disease, Alzheimer's disease, Cerebral Amyloid Angiopathy, and prion protein related disorders.

The vaccines of the present invention may be used in the prevention and/or treatment of amyloid related diseases, and in the manufacture of medicaments for  
20 preventing and/or treating amyloid-related diseases. A fibril peptide or protein can be derived from a fibril precursor protein known to be associated with certain forms of amyloid diseases, as described herein. Such precursor proteins include, but are not limited to, Serum Amyloid A protein (ApoSAA), immunoglobulin light chain, immunoglobulin heavy chain, ApoA1, transthyretin, lysozyme, fibrinogen  $\alpha$  chain,  
25 gelsolin, cystatin C, Amyloid beta protein precursor ( $\beta$ -APP), Beta<sub>2</sub> microglobulin, prion precursor protein (PrP), atrial natriuretic factor, keratin, islet amyloid polypeptide, a peptide hormone, and synuclein. Such precursors also include mutant proteins, protein fragments and proteolytic peptides of such precursors. In a preferred embodiment, the peptide is effective to induce an immune response

- directed against an epitope formed by an amyloidogenic protein or peptide, with respect to a fibril precursor protein. That is, as described in more detail herein, many fibril-forming peptides or proteins are fragments of such precursor proteins, such as those listed above. When such fragments are formed, such as by proteolytic
- 5 cleavage, epitopes may be revealed that are not present on the precursor and are therefore not immunologically available to the immune system when the fragment is a part of the precursor protein.

- In another embodiment, the peptide is effective to induce an immune response directed against an epitope formed by an amyloidogenic protein or peptide.
- 10 An amyloidogenic protein or peptide is synonymous with an amyloid peptide or protein, and encompasses a protein or peptide which is capable of forming fibrils, plaques, or amyloid deposits. The terms "A $\beta$ ," "A $\beta$  peptide", "β-amyloid peptide" and "Amyloidβ" peptide are synonymous, and refer to one or more peptide compositions of about 38-43 amino acids derived from Beta Amyloid Precursor
- 15 Protein (β-APP), as described herein. Disaggregated A $\beta$  means soluble, monomeric and oligomeric peptide units of A $\beta$ . One method to prepare monomeric A $\beta$  is to dissolve lyophilized peptide in neat DMSO with sonication. The resulting solution is centrifuged to remove any insoluble particulates. Aggregated A $\beta$  is a mixture of oligomers in which the monomeric units are held together by noncovalent bonds.
- 20 Furthermore, APP<sup>695</sup>, APP<sup>751</sup>, and APP<sup>770</sup> refer, respectively, to the 695, 751, and 770 amino acid residue long polypeptides encoded by the human APP gene. See Kang et al., *Nature* 325, 773 (1987); Ponte et al., *Nature* 331, 525 (1988); and Kitaguchi et al., *Nature* 331, 530 (1988). Amino acids within the human amyloid precursor protein (APP) are assigned numbers according to the sequence of the
- 25 APP770 isoform. Terms such as A $\beta$ 39, A $\beta$ 40, A $\beta$ 41, A $\beta$ 42 and A $\beta$ 43 refer, respectively, to an A $\beta$  peptide containing amino acid residues 1-39, 1-40, 1-41, 1-42 and 1-43. Preferably, A $\beta$  peptide contains amino acid residues 10-21. More preferably, the A $\beta$  peptide contains amino acid residues 13-21.

Accordingly, in one embodiment of the present invention, a vaccine is provided which is produced using a "non-self" peptide or protein synthesized from the unnatural D-configuration amino acids, to avoid the drawbacks of using "self" proteins. The peptides need not be aggregated to be operative or immunogenic, in  
5 contrast to the prior art vaccines. Thus, an "immunogenic peptide" or "immunogen" or "antigen" is a molecule that is capable of inducing an immunological response against itself upon administration to a patient, either in conjunction with, or in the absence of, an adjuvant. Such molecules include, for example, amyloidogenic peptides or fragments thereof conjugated to a carrier protein, such as keyhole limpet  
10 hemocyanin (KLH), C3d, polysaccharide, bovine serum albumin (BSA), Tetanus toxoid, heat shock protein (HSP), Ovalbumin or cholera toxin.

The term "immunological" or "immune" or "immunogenic" response refers to the development of a humoral (antibody mediated) and/or a cellular (mediated by antigen-specific T cells or their secretion products) response directed against an  
15 antigen in a vertebrate individual. Such a response can be an active response induced by administration of immunogen or a passive response induced by administration of antibodies or immune cells such as primed T-cells, B cells, macrophages NK or NKT cells, or primed dendritic cells which can act as antigen presenting cells. A cellular immune response is elicited by the presentation of polypeptide epitopes in  
20 association with Class I or Class II MHC molecules to activate antigen-specific CD4<sup>+</sup> T helper cells and/or CD8<sup>+</sup> cytotoxic T cells. The response may also involve activation of immune cells, such as monocytes, macrophages, NK cells, basophils, dendritic cells, astrocytes, microglia cells, eosinophils or other components of innate immunity. The presence of a cell-mediated immunological response can be  
25 determined by standard proliferation assays (CD4<sup>+</sup> T cells) or CTL (cytotoxic T lymphocyte) assays known in the art. The relative contributions of humoral and cellular responses to the protective or therapeutic effect of an immunogen can be distinguished by separately isolating immunoglobulin (IgG) and T-cell fractions

from an immunized first mammal and measuring a protective or therapeutic effect in a second subject.

The terms "polynucleotide" and "nucleic acid," as used interchangeably herein refer to a polymeric molecule having a backbone that supports bases capable of hydrogen bonding to typical polynucleotides, where the polymer backbone presents the bases in a manner to permit such hydrogen bonding in a sequence specific fashion between the polymeric molecule and a typical polynucleotide (e.g., single-stranded DNA). Such bases are typically inosine, adenosine, guanosine, cytosine, uracil and thymidine. Polymeric molecules include double and single stranded RNA and DNA, and backbone modifications thereof, for example, methylphosphonate linkages. The term "polypeptide" as used herein refers to a compound made up of a single chain of amino acid residues linked by peptide bonds. The term "protein" may be synonymous with the term "polypeptide" or may refer to a complex of two or more polypeptides. The term "peptide" also refers to a compound composed of amino acid residues linked by peptide bonds. Generally peptides are composed of 100 or fewer amino acids, while polypeptides or proteins have more than 100 amino acids. As used herein, the term "protein fragment" may also be read to mean a peptide.

Except as otherwise expressly defined herein, the abbreviations used herein for designating the amino acids and the protective groups are based on recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (*Biochemistry*, 1972, 11:1726-1732).

In another embodiment, there is provided a method for preventing and/or treating an amyloid-related disease in a subject, which features administering to the subject an antigenic amount of an all-D peptide which elicits production of antibodies against the all-D peptide, and elicits an immune response by the subject, therefore preventing fibrillogenesis, associated cellular toxicity and neurodegeneration, wherein the antibodies interact with at least one region of an

amyloid protein, a fibril protein or another non-amyloid protein which induces amyloidosis. A "fibril peptide" or "fibril protein" refers to a monomeric or oligomeric or aggregated form of a protein or peptide that forms fibrils present in amyloid plaques. Examples of such peptides and proteins are provided herein.

- 5 "Nonamyloid protein" containing formulations include, but are not limited to: compositions that produce immune responses against gelsolin fragments for treatment of hereditary systemic amyloidosis, mutant lysozyme protein (Alys), for treatment of a hereditary neuropathy, mutant alpha chain of fibrinogen (A $\alpha$ ) for a non-neuropathic form of amyloidosis manifest as renal disease, and mutant cystatin
- 10 C (Acys) for treatment of a form of hereditary cerebral angiopathy reported in Iceland. In addition, certain hereditary forms of prion disease (e.g., Creutzfeldt-Jacob disease (CJD), Gerstmann-Sträussler-Scheinker syndrome (GSS), and fatal familial insomnia (FFI)) are characterized by a mutant isoform of prion protein, PrP<sup>Sc</sup>. This protein can be used in therapeutic compositions for treatment and prevention
- 15 of deposition of PrP plaques, in accordance with the present invention. These vaccines may be used in the prevention and/or treatment of amyloid related diseases, and in the manufacture of medicaments for preventing and/or treating amyloid-related diseases.

- In a further embodiment of the invention, a vaccine for preventing and/or
- 20 treating an amyloid-related disease in a subject comprises at least one antibody or fragment thereof which interacts with amyloid proteins to prevent fibrillogenesis, wherein the antibodies are raised against an antigenic amount of an all-D peptide of such a protein, e.g.,  $\beta$  sheet region, GAG-binding site region, A $\beta$  (1-42, all-D) and macrophage adherence region A $\beta$  (10-16), immunogenic fragments thereof, protein
- 25 conjugates thereof, immunogenic derivative peptides thereof, immunogenic peptides thereof, and immunogenic peptidomimetics thereof, or a peptide which has a substantial identity to any of the above peptides.

The following terms are used to describe the sequence relationships between two or more polynucleotides: "substantial identity", "comparison window",

"sequence identity", "percentage of sequence identity", and "reference sequence." A "reference sequence" is a defined sequence used as a basis for a sequence comparison; a reference sequence may be a subset of a larger sequence, for example, as a segment of a full-length cDNA or gene sequence given in a sequence listing, or  
5 may comprise a complete cDNA or gene sequence. Generally, a reference sequence is at least 20 nucleotides in length, frequently at least 25 nucleotides in length, and often at least 50 nucleotides in length. Since two polynucleotides may each (1) comprise a sequence (i.e., a portion of the complete polynucleotide sequence) that is similar between the two polynucleotides, and (2) may further comprise a sequence  
10 that is divergent between the two polynucleotides, sequence comparisons between two (or more) polynucleotides are typically performed by comparing sequences of the two polynucleotides over a "comparison window" to identify and compare local regions of sequence similarity. A "comparison window", as used herein, refers to a conceptual segment of at least 20 contiguous nucleotide positions wherein a  
15 polynucleotide sequence may be compared to a reference sequence of at least 20 contiguous nucleotides and wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) of 20 percent or less as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Optimal  
20 alignment of sequences for aligning a comparison window may be conducted by the local homology algorithm of Smith and Waterman (1981) Adv. Appl. Math. 2: 482, by the homology alignment algorithm of Needleman and Wunsch (1970) J. Mol. Biol. 48: 443, by the search for similarity method of Pearson and Lipman (1988) Proc. Natl. Acad. Sci. (U.S.A.) 85: 2444, by computerized implementations of these  
25 algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Dr., Madison, Wis.), or by inspection, and the best alignment (i.e., resulting in the highest percentage of homology over the comparison window) generated by the various methods is selected.

The term "sequence identity" means that two polynucleotide sequences are identical (i.e., on a nucleotide-by-nucleotide basis) over the window of comparison. The term "percentage of sequence identity" is calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical nucleic acid base (e.g., A, T, C, G, U, or I) occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (i.e., the window size), and multiplying the result by 100 to yield the percentage of sequence identity. The terms "substantial identity" as used herein denotes a characteristic of a polynucleotide sequence, wherein the polynucleotide comprises a sequence that has at least 85 percent sequence identity, preferably at least 90 to 95 percent sequence identity, more usually at least 99 percent sequence identity as compared to a reference sequence over a comparison window of at least 20 nucleotide positions, frequently over a window of at least 25-50 nucleotides, wherein the percentage of sequence identity is calculated by comparing the reference sequence to the polynucleotide sequence which may include deletions or additions which total 20 percent or less of the reference sequence over the window of comparison.

As applied to polypeptides, the term "substantial identity" means that two peptide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap weights, share at least 80 percent sequence identity, preferably at least 90 percent sequence identity, more preferably at least 95 percent sequence identity or more (e.g., 99 percent sequence identity). Preferably, residue positions which are not identical differ by conservative amino acid substitutions. Conservative amino acid substitutions refer to the interchangeability of residues having similar side chains. For example, a group of amino acids having aliphatic side chains is glycine, alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is serine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of



amino acids having aromatic side chains is phenylalanine, tyrosine, and tryptophan; a group of amino acids having basic side chains is lysine, arginine, and histidine; and a group of amino acids having sulfur-containing side chains is cysteine and methionine. Preferred conservative amino acid substitution groups are: valine-  
5 leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, and asparagine-glutamine.

The term "antibody" or "immunoglobulin" is used to include intact antibodies and fragments thereof. An antibody can be a monoclonal or polyclonal antibody and can be made by recombinant techniques, collected from serum or  
10 ascites, or from hybridoma sources. Typically, fragments compete with the intact antibody from which they were derived for specific binding to an antigen fragment. Fragments are produced by recombinant DNA techniques, or by enzymatic or chemical cleavage of intact antibodies. Antigen binding fragments include Fab, Fab', F(ab')<sub>2</sub>, Fv, and single-chain antibodies, further including separate heavy chains  
15 or light chains. Antibody fragments are produced by recombinant DNA techniques, or by enzymatic or chemical separation of intact immunoglobulins. The term "antibody" encompasses one or more immunoglobulin chains that are chemically conjugated to, or expressed as, fusion proteins with other proteins. The term "antibody" also includes bispecific antibodies. A bispecific or bifunctional antibody  
20 is an artificial hybrid antibody having two different heavy/light chain pairs and two different binding sites. Bispecific antibodies can be produced by a variety of methods including fusion of hybridomas or linking of Fab' fragments. See, e.g., Songsivilai & Lachmann, *Clin. Exp. Immunol.* 79:315-321 (1990); Kostelny et al., *J. Immunol.* 148, 1547-1553 (1992).

25 As used herein, "Single-chain Fv" or "sFv" antibody fragments comprise the VH and VL domains of antibody, wherein these domains are present in a single polypeptide chain. Preferably, the Fv polypeptide further comprises a polypeptide linker between the VH and VL domains which enables the sFv to form the desired structure for antigen binding. For a review of sFv see, Pluckthun, The

Pharmacology of Monoclonal Antibodies. vol. 113. Rosenberg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994).

Specific binding between two entities means an affinity of at least  $10^6 \text{ M}^{-1}$ ,  $10^7 \text{ M}^{-1}$ ,  $10^8 \text{ M}^{-1}$ ,  $10^9 \text{ M}^{-1}$ , or  $10^{10} \text{ M}^{-1}$ . Affinities greater than  $10^8 \text{ M}^{-1}$  are preferred.

5 As discussed herein, minor variations in the amino acid sequences of antibodies or immunoglobulin molecules are contemplated as being encompassed by the present invention, providing that the variations in the amino acid sequence maintain at least 75%, more preferably at least 80%, 90%, 95%, and most preferably 99%. In particular, conservative amino acid replacements are contemplated. Conservative

10 replacements are those that take place within a family of amino acids that are related in their side chains. Genetically encoded amino acids are generally divided into families: (1) acidic=aspartate, glutamate; (2) basic=lysine, arginine, histidine; (3) non-polar=alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan; and (4) uncharged polar=glycine, asparagine, glutamine, cysteine, serine,

15 threonine, tyrosine. More preferred families are: serine and threonine are aliphatic-hydroxy family; asparagine and glutamine are an amide-containing family; alanine, valine, leucine and isoleucine are an aliphatic family; and phenylalanine, tryptophan, and tyrosine are an aromatic family. For example, it is reasonable to expect that an isolated replacement of a leucine with an isoleucine or valine, an aspartate with a

20 glutamate, a threonine with a serine, or a similar replacement of an amino acid with a structurally related amino acid will not have a major effect on the binding or properties of the resulting molecule, especially if the replacement does not involve an amino acid within a framework site. Whether an amino acid change results in a functional peptide can readily be determined by assaying the specific activity of the

25 polypeptide derivative. Assays are described in detail herein. Preferred amino- and carboxy-termini of fragments or analogs occur near boundaries of functional domains. Structural and functional domains can be identified by comparison of the nucleotide and/or amino acid sequence data to public or proprietary sequence databases. Preferably, computerized comparison methods are used to identify

30 sequence motifs or predicted protein conformation domains that occur in other

proteins of known structure and/or function. Methods to identify protein sequences that fold into a known three-dimensional structure are known. Bowie et al. *Science* 253:164 (1991). Thus, the foregoing examples demonstrate that those of skill in the art can recognize sequence motifs and structural conformations that may be used to

5 define structural and functional domains in accordance with the invention. Preferred amino acid substitutions are those which: (1) reduce susceptibility to proteolysis, (2) reduce susceptibility to oxidation, (3) alter binding affinity for forming protein complexes, (4) alter binding affinities, and (4) confer or modify other physicochemical or functional properties of such analogs. Analogs can include

10 various muteins of a sequence other than the naturally-occurring peptide sequence. For example, single or multiple amino acid substitutions (preferably conservative amino acid substitutions) may be made in the naturally-occurring sequence (preferably in the portion of the polypeptide outside the domain(s) forming intermolecular contacts). A conservative amino acid substitution should not

15 substantially change the structural characteristics of the parent sequence (e.g., a replacement amino acid should not tend to break a helix that occurs in the parent sequence, or disrupt other types of secondary structure that characterizes the parent sequence). Examples of art-recognized polypeptide secondary and tertiary structures are described in *Proteins, Structures and Molecular Principles* (Creighton, Ed., W.

20 H. Freeman and Company, New York (1984)); *Introduction to Protein Structure* (C. Branden and J. Tooze, eds., Garland Publishing, New York, N.Y. (1991)); and Thornton et al. *Nature* 354:105 (1991), which are each incorporated herein by reference.

25 As used herein, "Humanized" forms of non-human (e.g., rodent) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', F(ab')<sub>2</sub>, or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in

30 which residues from a hypervariable region of the recipient are replaced by residues

from a hypervariable region of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity, and capacity. In some instances, framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies  
5 may comprise residues which are found neither in the recipient antibody nor in the donor antibody. These modifications are made to further refine and maximize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human  
10 immunoglobulin and all or substantially all of the FRs are those of a human immunoglobulin sequence. The humanized antibody optionally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see. Jones et al., *Nature*, 322:522-525 (1986); Reichmarm et al., *Nature*, 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.*,  
15 2:593-596 (1992).

The term "conjugate" or "conjugated to" is intended to refer to joined together, coupled, bonded and/or fused. A "protein conjugate" or "conjugated protein" is generally intended to refer to a compound of a protein with a non-protein. In addition to the amino acids, a "conjugated protein" contains permanently  
20 associated chemical components. The non-amino acid part of the conjugated protein is generally referred to as its prosthetic group. Conjugated proteins are classified on the basis of the chemical nature of their prosthetic groups (*Lehninger Principles of Biochemistry*, 3d ed. Worth Publishers 2000), which is incorporated by reference herein. In a conjugate vaccine, an antigenic molecule is covalently linked to a  
25 "carrier" protein or polypeptide. The linkage serves to increase the antigenicity of the conjugated molecule. Methods for forming conjugate vaccines from an antigenic molecule and a "carrier" protein or polypeptide are known in the art (Jacob, C. O, et al., *Eur. J. Immunol.* 16:1057-1062 (1986); Parker J. M. R. et al., In: *Modern Approaches to Vaccines*, Chanock, R. M. et al., eds, pp. 133-138, Cold Spring  
30 Harbor Laboratory, Cold Spring Harbor, N.Y. (1983); Zurawski, V. R, et al., J.

Immunol. 121:122-129 (1978); Klipstein, F. A, et al., Infect. Immun. 37:550-557 (1982); Bessler, W. G, Immunobiol. 170:239-244 (1985); Posnett, D. N, et al., J. Biol. Chem. 263:1719-1725 (1988); Ghose, A. C, et al., Molec. Immunol. 25:223-230 (1988); all of which references are incorporated herein by reference).

- 5 Conjugation of a protein and a polysaccharide can provide other advantageous results. For example, it has been found that protein-polysaccharide conjugates enhance the antibody response not only to the polysaccharide component, but also to the protein component. This effect is described, for example, in the dual conjugate patent application of Mond and Lees, U.S. Pas Nos. 5,955,079 and 5,585,100, both of which are incorporated herein by reference. This effect also is described in A. Lees, et al., "Enhanced Immunogenicity of Protein-Dextran Conjugates: I. Rapid Stimulation of Enhanced Antibody Responses to Poorly Immunogenic Molecules," Vaccine, Vol. 12, No. 13, (1994), pp. 1160-1166. This article is entirely incorporated herein by reference. Examples of carrier proteins include, but are not limited to
- 10 of which are incorporated herein by reference. This effect also is described in A. Lees, et al., "Enhanced Immunogenicity of Protein-Dextran Conjugates: I. Rapid Stimulation of Enhanced Antibody Responses to Poorly Immunogenic Molecules," Vaccine, Vol. 12, No. 13, (1994), pp. 1160-1166. This article is entirely incorporated herein by reference. Examples of carrier proteins include, but are not limited to
- 15 keyhole limpet hemocyanin (KLH), C3d, polysaccharide, bovine serum albumin (BSA), Tetanus toxoid, heat shock protein (HSP), Ovalbumin or cholera toxin.

In another embodiment, there is provided a method for preventing and/or treating an amyloid-related disease in a subject which comprises administering a pharmaceutical composition comprising a combination of at least two antibodies, or

20 fragments thereof that bind to two or more portions of a fibril protein and/or a nonamyloid protein. In another embodiment the pharmaceutical composition further comprises immune cells, more preferably primed lymphocytes.

A "pharmaceutical composition" refers to a chemical or biological composition suitable for administration to a mammalian individual, preferably a

25 human. Such compositions may be specifically formulated for administration via one or more of a number of routes, including but not limited to, oral, parenteral, intravenous, intraarterial, subcutaneous, intranasal, sublingual, intraspinal, intracerebroventricular, transdermal and the like. A "pharmaceutical excipient" or a "pharmaceutically acceptable excipient" is a carrier, usually a liquid, in which an

active therapeutic peptide is formulated. The excipient generally does not provide any pharmacological activity to the formulation, though it may provide chemical and/or biological stability, release characteristics, and the like. Exemplary formulations can be found, for example, in Remington's Pharmaceutical Sciences, 5 19<sup>th</sup> Ed., Grennaro, A., Ed., 1995.

Still in a further embodiment, there is provided a method for preventing and/or treating an amyloid-related disease in a subject, which comprises administering to the subject an antigenic amount of an all-D peptide which, may further interact with at least one region of an amyloid protein, *e.g.*,  $\beta$  sheet region, 10 GAG-binding site region, A $\beta$  (1-42) and macrophage adherence region A $\beta$  (10-16), immunogenic fragments thereof, protein conjugates thereof, immunogenic derivative peptides thereof, immunogenic peptides thereof, and immunogenic peptidomimetics thereof, wherein the compound elicits an immune response by the subject and therefore prevents fibrillogenesis.

15 According to another related aspect, the invention includes a method of preventing or treating a disorder characterized by amyloid deposition in a mammalian subject. In accordance with this aspect of the invention, the subject is given a dosage of a peptide effective to produce an immune response against an amyloid peptide characteristic of the amyloid disorder from which the subject 20 suffers. Essentially, the methods include administering pharmaceutical compositions containing immunogenic amyloid peptides specific to the disorder, such as those described above. Such methods are further characterized by their effectiveness in inducing immunogenic responses in the subject. According to a preferred embodiment, the method is effective to produce an immunological response that is 25 characterized by a serum titer of at least 1:1000 with respect to the amyloid peptide against which the immunogenic peptide is directed. In yet a further preferred embodiment, the serum titer is at least 1:5000 with respect to the amyloid component. According to a related embodiment, the immune response is characterized by a serum amount of immunoreactivity corresponding to greater than

about four times higher than a serum level of immunoreactivity measured in a pre-treatment control serum sample. This latter characterization is particularly appropriate when serum immunoreactivity is measured by ELISA techniques, but can apply to any relative or absolute measurement of serum immunoreactivity.

- 5 According to a preferred embodiment, the immunoreactivity is measured at a serum dilution of about 1:100 to 1:10,000 to determine antibody titer.

According to a still further related aspect, the invention includes a method of determining the prognosis of a patient undergoing treatment for an amyloid disorder. Here, patient serum amount of immunoreactivity against an amyloid peptide  
10 characteristic of the selected disorder is measured, and a patient serum amount of immunoreactivity of at least four times a baseline control level of serum immunoreactivity is indicative of a prognosis of improved status with respect to the particular amyloid disorder. According to preferred embodiments, the amount of immunoreactivity against the selected amyloid peptide present in the patient serum is  
15 characterized by a serum titer of at least about 1:1000, or at least 1:5000, with respect to the amyloid peptide.

"Passive immunization" as used herein refers to the administration of antibodies, fragments thereof, or immune cells, i.e. T-cells B-cells, NK cells, NKT cells, dendritic cells, macrophages, basophils, monocytes, or components of the  
20 complement pathway, to an individual in order to confer immunity. Components of the complement pathway can be conjugated to proteins to enhance the innate immune response in combination with active or passive immunization. Immunoglobulins (Ig) obtained from human blood may contain antibodies to a variety of agents depending on the pool of human plasma used in preparation. Specific  
25 immunoglobulins are obtained from plasma from donors with high levels of antibodies to specific antigens, or donors immunized to produce such a response (Immunization, Cecil Textbook of Medicine, 19<sup>th</sup> ed. Vol. 1, W.B. Saunders Company 1992; Harrison's Principles of Internal Medicine, 14<sup>th</sup> ed, McGraw Hill, 1998). Humanized monoclonal antibodies to sequester amyloid- $\beta$  peptide in plasma,

brain and cerebrospinal fluid to prevent accumulation of the amyloid- $\beta$  peptide within the brain and the cerebrovasculature is described in WO01/62801, which is incorporated herein by reference. EP0613007 describes antibodies having specificity for  $\beta$ -amyloid peptide which is predominantly in a  $\beta$ -sheet conformation. Such  
5 antibodies are useful for the invention as described herein.

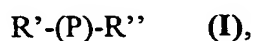
According to one embodiment of the invention, methods and compositions are provided for passive immunization of a mammal in order to prevent or treat amyloid diseases. According to this aspect of the invention, patients are given an effective dosage of an antibody or fragment thereof, that specifically binds to a  
10 selected region of amyloid peptide, preferably a region close to or within the central core region of the peptide which is responsible for  $\beta$ -sheet formation or are given immune cells, preferably primed immune cells or antigen presenting cells. In general, such antibodies are selected for their abilities to specifically bind the various proteins, peptides, and components described with respect to the pharmaceutical  
15 compositions and methods described in the preceding paragraphs of this section. According to a related embodiment, such methods and compositions may include combinations of antibodies that bind at least two amyloid components. In general, pharmaceutical compositions are administered to provide a serum amount of immunoreactivity against the target amyloid peptide that is at least about four times  
20 higher than a serum level of immunoreactivity against the component measured in a control serum sample. The antibodies may also be administered with a carrier, as described herein. In general, in accordance with this aspect of the invention, such antibodies will be administered (or formulated for administration) peritoneally, orally, intranasally, subcutaneously, intramuscularly, transdermally, topically or  
25 intravenously, but can be administered or formulated for administration by any pharmaceutically effective route (i.e., effective to produce the indicated therapeutic levels, as set forth above and herein).

The term "epitope" or "antigenic determinant" refers to a site on an antigen to which an antibody or antibody binding fragment specifically binds or to which B



and/or T cells respond. B-cell epitopes can be formed both from contiguous amino acids or noncontiguous amino acids juxtaposed by tertiary folding of a protein. Epitopes formed from contiguous amino acids are typically retained on exposure to denaturing solvents whereas epitopes formed by tertiary folding are typically lost on treatment with denaturing solvents. An epitope typically includes at least 3, and more usually, at least 5 or 8-10 amino acids in a unique spatial conformation. Methods of determining spatial conformation of epitopes include, for example, x-ray crystallography and 2-dimensional nuclear magnetic resonance. See, e.g., *Epitope Mapping Protocols in Methods in Molecular Biology*, Vol. 66, Glenn E. Morris, Ed. (1996). Antibodies that recognize the same epitope can be identified in a simple immunoassay showing the ability of one antibody to block the binding of another antibody to a target antigen. L-cells recognize continuous epitopes of about nine amino acids for CD8 cells or about 13-15 amino acids for CD4 cells. T cells that recognize the epitope can be identified by *in vitro* assays that measure antigen-dependent proliferation, as determined by  $^3\text{H}$ -thymidine incorporation by primed T cells in response to an epitope (Burke et al., *J. Inf Dis.* 170, 1110-19 (1994)), by antigen-dependent killing (cytotoxic T lymphocyte assay, Tigges et al., *J. Immunol.* 156, 3901-3910) or by cytokine secretion.

As used herein, the term "compound" refers to a peptide of the present invention or a pharmaceutically acceptable composition containing a peptide according to the present invention. In a preferred embodiment of the present invention, the compound is a compound of Formula I:



wherein

P is an all-D peptide of an amyloid protein, e.g.,  $\beta$  sheet region, GAG-binding site region, A $\beta$  (1-42, all-D), and macrophage adherence region (10-16, all-D), immunogenic fragments thereof, immunogenic derivatives thereof, protein conjugates thereof,

immunogenic peptides thereof, and immunogenic peptidomimetics thereof;

R' is an N-terminal substituent, *e.g.* :

- hydrogen;
- 5       ▪ lower alkyl groups, *e.g.*, acyclic or cyclic having 1 to 8 carbon atoms, without or with functional groups, *e.g.*, carboxylate, sulfonate and phosphonate;
- aromatic groups;
- heterocyclic groups; and
- 10       ▪ acyl groups, *e.g.*, alkylcarbonyl, arylcarbonyl, sulfonyl and phosphonyl groups; and

R'' is a C-terminal substituent, *e.g.*, hydroxy, alkoxy, aryloxy, unsubstituted or substituted amino groups.

In an embodiment, R' and R'' are identical or different, wherein the alkyl or  
15       aryl groups of R' and R'' are further substituted with functionalities such as halide (*e.g.*, F, Cl, Br, and I), hydroxyl, alkoxyl, aryloxy, hydroxycarbonyl, alkoxycarbonyl, aryloxy carbonyl, carbamyl, unsubstituted or substituted amino, sulfo or alkyloxysulfonyl, phosphono or alkoxyphosphonyl groups.

When the compound has an acid functional group, it can be in the form of a  
20       pharmaceutically acceptable salt or ester. When the compound has a basic functional group, it can be in the form of a pharmaceutically acceptable salt.

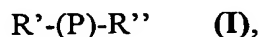
In one embodiment, P is a peptide capable of interacting with at least one region of an amyloid protein.

In a preferred embodiment of the present invention, the subject is a human  
25       being.

In yet another embodiment of the present invention, the amyloid related disease may be Alzheimer's disease.

In still another embodiment of the present invention, the amyloid related disease may be Cerebral Amyloid Angiopathy (CAA).

In another embodiment of the present invention, there is provided a method for preventing and/or treating an amyloid related disease in a subject, comprising  
 5 administering to the subject an antigenic amount of a compound of Formula I:



wherein

10 P is an all-D peptide of an amyloid protein, *e.g.*,  $\beta$  sheet region, GAG-binding site region, A $\beta$  (1-42, all-D), and macrophage adherence region (10-16, all-D), immunogenic fragments thereof, immunogenic derivatives thereof, protein conjugates thereof, immunogenic peptides thereof, and immunogenic peptidomimetics thereof;

15 R' is an N-terminal substituent selected from the group consisting of:

- hydrogen;
- lower alkyl groups, *e.g.*, acyclic or cyclic having 1 to 8 carbon atoms, without or with functional groups, *e.g.*, carboxylate, sulfonate and phosphonate;
- 20 ▪ aromatic groups;
- heterocyclic groups; and
- acyl groups, *e.g.*, alkylcarbonyl, arylcarbonyl, sulfonyl and phosphonyl groups; and

R'' is a C-terminal substituent, *e.g.*, hydroxy, alkoxy, aryloxy, unsubstituted or substituted amino groups.

In accordance with this method, the compound elicits an immune response by the subject preventing fibrillogenesis, neurodegeneration or cellular toxicity.

5 In accordance with a preferred embodiment of the present invention, there is provided a vaccine for preventing and/or treating an amyloid-related disease in a subject, comprising an antigenic amount of an all-D peptide which may further interact with at least one region of an amyloid protein, *e.g.*,  $\beta$  sheet region (16-21), GAG-binding site region (13-16), and macrophage adherence region (10-16, all-D),  
10 immunogenic fragments thereof, protein conjugates thereof, immunogenic derivative peptides thereof, immunogenic peptides thereof, and immunogenic peptidomimetics thereof, wherein the compound elicits an immune response by the subject or prevents fibrillogenesis or favors clearance of amyloid protein prior to fibril formation. In one aspect, the vaccine alters soluble A $\beta$  levels in the plasma of a  
15 mammal thereby decreasing soluble A $\beta$  levels in the brain of a mammal, and thereby preventing the formation of fibril plaques.

In another embodiment, some methods entail passive immunization against soluble A $\beta$  or fibrillary A $\beta$ . In one aspect, the invention provides a method for preventing and/or treating an amyloid-related disease in a first mammal in need  
20 thereof, comprising administering an antigenic amount of an all-D amyloid- $\beta$  peptide to a second mammal, wherein said all-D amyloid- $\beta$  peptide induces an immune response against the amyloid- $\beta$  peptide in the second mammal, recovering the antibodies or immune cells from the second mammal, and providing a therapeutically or prophylactically effective dose of the antibodies or immune cells  
25 to the first mammal. In this embodiment the all-D amyloid- $\beta$  peptide has an amino acid sequence that is at least 80% identical to a sequence selected from the group consisting of SEQ ID NOS: 1-70. Other reactive epitopes of A $\beta$  useful for the generating antibodies or immune cells according to the present invention include an

epitope within residues 1-42 of A $\beta$ , an epitope within residues 16-21 of A $\beta$ , an epitope within residues 13-21 of A $\beta$ , an epitope within residues 10-21 of A $\beta$ , an epitope within residues 10-16 of A $\beta$ , an epitope within residues 25-35 of A $\beta$ , and an epitope comprising a free N- or C-terminal residue of A $\beta$ . In one aspect, passive immunization against A $\beta$  is effectuated by transference of an immune cell, i.e., a primed lymphocyte such as a B or T cell, from a second mammal actively immunized against A $\beta$  as described, to a first mammal thereby ameliorating or preventing A $\beta$  related disorders in the first mammal. In another aspect, passive immunization against A $\beta$  is effectuated by transference of an antibody or fragment thereof capable of binding specifically to A $\beta$ . In yet another aspect a human, humanized or chimeric antibody is administered to the first mammal thereby ameliorating or preventing A $\beta$  related disorders.

In one embodiment, the present invention is directed to a method for preventing and/or treating an amyloid-related disease in a subject, comprising: administering to the subject an antigenic amount of an all-D amyloid- $\beta$  peptide, wherein said all-D amyloid- $\beta$  peptide induces an immune response by said subject against said amyloid- $\beta$  peptide, and wherein said all-D amyloid- $\beta$  peptide has an amino acid sequence that is at least 80% identical to a sequence selected from the group consisting of SEQ ID NOS: 1-70 or least one antibody or fragment that specifically binds to an epitope within residues 1-42 of A $\beta$ , an epitope within residues 16-21 of A $\beta$ , an epitope within residues 13-21 of A $\beta$ , an epitope within residues 10-21 of A $\beta$ , an epitope within residues 10-16 of A $\beta$ , an epitope within residues 25-35 of A $\beta$ , and an epitope comprising a free N- or C-terminal residue of A $\beta$ .

In one embodiment, the present invention is directed to altering the serum levels of A $\beta$  in a mammal in need thereof, for example a human, by administering a therapeutic or prophylactic amount of at least one antibody or fragment that specifically binds to an epitope within residues 1-42 of A $\beta$ , an epitope within residues 16-21 of A $\beta$ , an epitope within residues 13-21 of A $\beta$ , an epitope within

residues 10-21 of A $\beta$ , an epitope within residues 10-16 of A $\beta$ , an epitope within residues 25-35 of A $\beta$ , and an epitope comprising a free N- or C-terminal residue of A $\beta$ . In one aspect, altering serum A $\beta$  levels in the mammal alters the levels of soluble A $\beta$  or fibril A $\beta$  in the mammal. In yet another aspect, altering serum A $\beta$  levels in the mammal alters the levels of soluble or fibril A $\beta$  levels in the brain of the mammal. In another aspect altering serum A $\beta$  levels in the mammal favors the clearance of A $\beta$  levels in the brain of a mammal.

The methods can be used on both asymptomatic patients and those currently showing symptoms of disease. The antibody administered in such methods can be a human, humanized, chimeric or nonhuman antibody, or fragment thereof and can be from monoclonal or polyclonal sources, obtained from a mammal, or produced by recombinant techniques. In some methods, the antibody is prepared from a human immunized with A $\beta$  peptide. In other methods, a humanized antibody is administered.

In some methods, the antibody is administered with a pharmaceutical carrier as a pharmaceutical composition. In some methods, antibody is administered at a dosage of 0.0001 to 100 mg/kg, preferably, at least 1 mg/kg body weight antibody. In some methods, the antibody is administered in multiple dosages over a prolonged period, for example, of at least six months. In some methods, the antibody is administered as a sustained release composition. The antibody can be administered, for example, intraperitoneally, orally, subcutaneously, intracranially, intramuscularly, topically, intranasally, transdermally or intravenously.

Other features and advantages of the invention will be apparent from the following detailed description and claims.

## BRIEF DESCRIPTION OF THE DRAWING

FIG. 1 illustrates the targeted sites for the antigenic fragments.

FIG. 2 illustrates the effect of 1 mg/ml of polyclonal antibodies raised against D and L forms of A $\beta$ (16-21) on fibrillogenesis.

FIG. 3 illustrates the effect of 0.5 mg/ml of polyclonal antibodies raised  
5 against D and L forms of A $\beta$ (16-21) on fibrillogenesis.

FIGs. 4A to 4C illustrate electron micrographs showing the effect of anti-D KLVFFA peptide antibodies (FIG. 4B) and anti-L KLVFFA peptide antibodies (FIG. 4C) with respect to a control (FIG. 4A) on fibrillogenesis.

FIGs. 5A to 5D illustrate the immunohistochemistry of anti-D KLVFFA on  
10 aggregated A $\beta$  peptide in brain sections of retrosplenial cortex (FIG. 5A) and parietal cortex (FIG. 5C) and the histochemistry (Thioflavin S assay) of anti-D KLVFFA on aggregated A $\beta$  peptide in the same brain sections of retrosplenial cortex (FIG. 5B) and parietal cortex (FIG. 5D).

FIGs. 6A to 6D illustrate the immunohistochemistry of anti-L KLVFFA  
15 antibodies on aggregated A $\beta$  peptide in brain sections of parietal cortex (FIG. 6A) and entorhinal cortex (FIG. 6C) and the histochemistry (Thioflavin S assay) of anti-L KLVFFA antibodies on aggregated A $\beta$  peptide in the same brain sections of parietal cortex (FIG. 6B) and entorhinal cortex (FIG. 6D).

FIG. 7 illustrates the response of rabbits to KLH-conjugated all-L and all-D  
20 KLVFFA.

FIGs. 8A and 8B illustrate the reduction of A $\beta$ 40 level, soluble and insoluble fraction in the brain of TgCRND8 mice immunized with D13-21-KLH.

Fig. 9 illustrates the reduction of A $\beta$ 42 level, soluble fraction in the brain of TgCRND8 mice immunized with D13-21-KLH.

FIGs. 10A and 10B illustrate the reduction of total A $\beta$  level (A $\beta$ 40 and A $\beta$ 42) in the brain of TgCRND8 mice immunized with D13-21-KLH.

## DETAILED DESCRIPTION OF THE INVENTION

5           Amyloid diseases or amyloidoses include a number of disease states having a wide variety of outward symptoms. These disorders have in common the presence of abnormal extracellular deposits of protein fibrils, known as “amyloid deposits” or “amyloid plaques” that are usually about 10-100nm in diameter and are localized to specific organs or tissue regions. Such plaques are composed primarily of a naturally  
10           occurring soluble protein or peptide which aggregate in insoluble fibrillar deposits. These insoluble deposits are composed of generally lateral aggregates of fibrils that are approximately 10-15 nm in diameter. Amyloid fibrils produce a characteristic apple green birefringence in polarized light, when stained with Congo Red dye.

          The peptides or proteins forming the plaque deposits are often produced from  
15           a larger precursor protein. More specifically, the pathogenesis of amyloid fibril deposits generally involves proteolytic cleavage of an “abnormal” precursor protein into fragments. These fragments generally aggregate into anti-parallel  $\beta$ -pleated sheets; however, certain undegraded forms of precursor protein have been reported to aggregate and form fibrils in familial amyloid polyneuropathy (variant  
20           transthyretin fibrils) and dialysis-related amyloidosis ( $\beta_2$  microglobulin fibrils) (Tan, S.Y. and Pepys, M.B. Amyloidosis. Histopathology 25: 403-414 (1994)). Specifically, the A $\beta$  (16-21) site is known to play an important role in initiating the harmful process of A $\beta$  peptide amyloidogenesis. It is also known that when these peptides are made from D-amino acids, they retain their ability to interact with the  
25           natural all-L-homologous sequence, thereby preventing amyloidogenesis. Other amyloid proteins which may be used in the present invention include, without



limitation, the beta sheet regions of IAPP (e.g. 24-29, all-D),  $\beta$ 2-microglobulin, amyloid A protein, and prion-related proteins.

The disorders are traditionally classified on the basis of the major fibril components forming the plaque deposits, as discussed below. However, without  
5 being restricted to theory, there is evidence supporting  $A\beta$  can be transported back and forth between the brain and the blood (Gherzi-Egea, J., et al., J. Neurochem., 67:880-883, (1996)) and that  $A\beta$  in plaques (insoluble or fibrillar  $A\beta$ ) is in equilibrium with soluble  $A\beta$  (Kawarabayashi, T., et al., J. Neurosci. 21:372-381, (2001), both incorporated herein by reference in their entirety. Thus, the  
10 identification of a disorder based on the major fibril component is provided for convenience, and amelioration of the disease can be effectuated according to the compositions and methods described herein by targeting either the fibrillar  $A\beta$  or the soluble  $A\beta$ . The targeting of either the fibrillar  $A\beta$  or the soluble  $A\beta$  can be achieved by active or passive immunization as described herein and in PCT  
15 publications WO 01/62801, WO 01/90182, WO 01/18169, WO 00/77178, WO 00/72880, WO 00/72876, WO 99/60024 and WO 99/27944, each incorporated by reference in its entirety. Levels of targeted  $A\beta$ , specifically levels of the soluble  $A\beta$  can be determined by methods known in the art and disclosed herein (*see*, U.S. patents 5,766,846, 5,837,672, and 5,593,846, each incorporated by reference in their  
20 entirety). These levels can be monitored during the course of prophylactic or therapeutic treatments. A therapeutic endpoint is reached when the disease state is reduced.

### **Amyloid Diseases**

The present invention is based on the discovery that amyloid diseases can be  
25 treated by administering peptides that serve to stimulate an immune response against a component or components of the various disease-specific amyloid deposits. The sections below serve to exemplify major forms of amyloidosis and are not intended to limit the invention.

*AA (reactive) Amyloidosis*

Generally, AA amyloidosis is a manifestation of a number of diseases that provoke a sustained acute phase response. Such diseases include chronic inflammatory disorders, chronic local or systemic microbial infections, and  
5 malignant neoplasms.

AA fibrils are generally composed of 8000 dalton fragments (AA peptide or protein) formed by proteolytic cleavage of serum amyloid A protein (ApoSAA), a circulating apolipoprotein which is present in HDL complexes and which is synthesized in hepatocytes in response to such cytokines as IL-1, IL-6 and TNF.  
10 Deposition can be widespread in the body, with a preference for parenchymal organs. The spleen is usually a deposition site, and the kidneys may also be affected. Deposition is also common in the heart and gastrointestinal tract.

AA amyloid diseases include, but are not limited to inflammatory diseases, such as rheumatoid arthritis, juvenile chronic arthritis, ankylosing spondylitis, psoriasis, psoriatic arthropathy, Reiter's syndrome, Adult Still's disease, Behcet's  
15 syndrome, and Crohn's disease. AA deposits are also produced as a result of chronic microbial infections, such as leprosy, tuberculosis, bronchiectasis, decubitus ulcers, chronic pyelonephritis, osteomyelitis, and Whipple's disease. Certain malignant neoplasms can also result in AA fibril amyloid deposits. These include such  
20 conditions as Hodgkin's lymphoma, renal carcinoma, carcinomas of gut, lung and urogenital tract, basal cell carcinoma, and hairy cell leukemia.

*AL Amyloidoses*

AL amyloid deposition is generally associated with almost any dyscrasia of the B lymphocyte lineage, ranging from malignancy of plasma cells (multiple  
25 myeloma) to benign monoclonal gammopathy. At times, the presence of amyloid deposits may be a primary indicator of the underlying dyscrasia.

- Fibrils of AL amyloid deposits are composed of monoclonal immunoglobulin light chains or fragments thereof. More specifically, the fragments are derived from the N-terminal region of the light chain (kappa or lambda) and contain all or part of the variable ( $V_L$ ) domain thereof. Deposits generally occur in the mesenchymal tissues, causing peripheral and autonomic neuropathy, carpal tunnel syndrome, macroglossia, restrictive cardiomyopathy, arthropathy of large joints, immune dyscrasias, myelomas, as well as occult dyscrasias. However, it should be noted that almost any tissue, particularly visceral organs such as the heart, may be involved.

#### 10 *Hereditary Systemic Amyloidoses*

- There are many forms of hereditary systemic amyloidoses. Although they are relatively rare conditions, adult onset of symptoms and their inheritance patterns (usually autosomal dominant) lead to persistence of such disorders in the general population. Generally, the syndromes are attributable to point mutations in the precursor protein leading to production of variant amyloidogenic peptides or proteins. Table 1 summarizes the fibril composition of exemplary forms of these disorders.

**Table 1**

<i>Fibril Peptide/Protein</i>	<i>Genetic variant</i>	<i>Clinical Syndrome</i>
Transthyretin and fragments (ATTR)	Met30, many others	Familial amyloid polyneuropathy (FAP), (Mainly peripheral nerves)
Transthyretin and fragments (ATTR)	Thr45, Ala60, Ser84, Met111, Ile122	Cardiac involvement predominant without neuropathy
N-terminal fragment of Apolipoprotein A1 (apoA1)	Arg26	Familial amyloid polyneuropathy (FAP), (mainly peripheral nerves)
N-terminal fragment of Apolipoprotein A1 (ApoA1)	Arg26, Arg50, Arg 60, others	Ostertag-type, non-neuropathic (predominantly visceral involvement)

Lysozyme (Alys)	Thr56, His67	Ostertag-type, non-neuropathic (predominantly visceral involvement)
Fibrogen $\nabla$ chain fragment	Leu554, Val 526	Cranial neuropathy with latic corneal dystrophy
Gelsolin fragment (Agel)	Asn187, Tyr187	Cranial neuropathy with lattice cornealk dystrophy
Cystatin C fragment	Glu68	Hereditary cerebral hemmorrhage (cerebral amyloid angiopathy) – Icelandic type
$\beta$ -amyloid protein ( $a\beta$ ) derived from Amyloid Precursor Protein (APP)	Gln693	Hereditary cerebral hemmorrhage (cerebral amyloid angiopathy) – Dutch type
$\beta$ -amyloid protein ( $a\beta$ ) derived from Amyloid Precursor Protein (APP)	Ile717, Phe717, Gly717	Familial Alzheimer's Disease
$\beta$ -amyloid protein ( $a\beta$ ) derived from Amyloid Precursor Protein (APP)	Asn670, Leu671	Familial Dementia – probably Alzheimer's Disease
Prion Protein (PrP) derived from Prp precursor protein 51-91 insert	Leu102, Val167, Asn178, Lys200	Familial Creutzfeldt-Jakob disease; Gerstmann-Sträussler-Scheinker syndrome (hereditary spongiform encephalopathies, prion diseases)
AA derived from Serum amyloid A protein (ApoSAA)		Familial Mediterranean fever, predominant renal involvement (autosomal recessive)
AA derived from Serum amyloid A protein (ApoSAA)		Muckle-Well's syndrome, nephropathy, deafness, urticaria, limb pain
Unknown		Cardiomyophathy with persistent atrial standstill
Unknown		Cutaneous deposits (bullous, papular, pustuloderma)

\*Data derived from Tan & Pepys, 1994, supra.

The data provided in Table 1 are exemplary and are not intended to limit the scope of the invention. For example, more than 40 separate point mutations in the transthyretin gene have been described, all of which give rise to clinically similar forms of familial amyloid polyneuropathy.

5           Transthyretin (TTR) is a 14 kilodalton protein that is also sometimes referred to as prealbumin. It is produced by the liver and choroid plexus, and it functions in transporting thyroid hormones and vitamin A. At least 50 variant forms of the protein, each characterized by a single amino acid change, are responsible for various forms of familial amyloid polyneuropathy. For example, substitution of  
10   proline for leucine at position 55 results in a particularly progressive form of neuropathy; substitution of methionine for leucine at position 111 resulted in a severe cardiopathy in Danish patients. Amyloid deposits isolated from heart tissue of patients with systemic amyloidosis have revealed that the deposits are composed of a heterogeneous mixture of TTR and fragments thereof, collectively referred to as  
15   ATTR, the full length sequences of which have been characterized. ATTR fibril components can be extracted from such plaques and their structure and sequence determined according to the methods known in the art (e.g., Gustavsson, A., *et al.*, Laboratory Invest. 73: 703-708, 1995; Kametani, F., *et al.*, Biochem. Biophys. Res. Commun. 125: 622-628, 1984; Pras, M., *et al.*, PNAS 80: 539-42, 1983).

20           Persons having point mutations in the molecule apolipoprotein AI (e.g., Gly→Arg26; Trp 4→Arg50; Leu→4 Arg60) exhibit a form of amyloidosis ("Östertag type") characterized by deposits of the protein apolipoprotein AI or fragments thereof (AApoAI). These patients have low levels of high density lipoprotein (HDL) and present with a peripheral neuropathy or renal failure.

25           A mutation in the alpha chain of the enzyme lysozyme (e.g., Ile→Thr56 or Asp→His57) is the basis of another form of Östertag-type non-neuropathic hereditary amyloid reported in English families. Here, fibrils of the mutant lysozyme protein (Alys) are deposited, and patients generally exhibit impaired renal function.

This protein, unlike most of the fibril-forming proteins described herein, is usually present in whole (unfragmented) form (Benson, M.D., et al. CIBA Fdn. Symp. 199: 104-131, 1996).

5         $\beta$ -amyloid peptide ( $A\beta$ ) is a 39-43 amino acid peptide derived by proteolysis from a large protein known as  $\beta$  Amyloid Precursor protein ( $\beta$ APP). Mutations in  $\beta$ APP result in familial forms of Alzheimer's disease, Down's syndrome and/or senile dementia, characterized by cerebral deposition of plaques composed of  $A\beta$  fibrils and other components, which are described in further detail below. Known mutations in APP associated with Alzheimer's disease occur proximate to the

10        cleavage sites of  $\beta$  or gamma-secretase, or within  $A\beta$ . For example, position 717 is proximate to the site of gamma-secretase cleavage of APP in its processing to  $A\beta$ , and positions 670/671 are proximate to the site of  $\beta$ -secretase cleavage. Mutations at any of these residues may result in Alzheimer's disease, presumably by causing an increase the amount of the 42/43 amino acid form of  $A\beta$  generated from APP. The

15        structure and sequence of  $A\beta$  peptides of various lengths are well known in the art. Such peptides can be made according to methods known in the art (e.g., Glenner and Wong, Biochem Biophys. Res. Comm. 129: 885-890, 1984; Glenner and Wong, Biochem Biophys. Res. Comm. 122: 113 1-1135, 1984). In addition, various forms of the peptides are commercially available.

20        Synuclein is a synapse-associated protein that resembles an apolipoprotein and is abundant in neuronal cytosol and presynaptic terminals. A peptide fragment derived from alpha-synuclein, termed NAC, is also a component of amyloid plaques of Alzheimer's disease. (Clayton, et al., 1998). This component also serves as a target for immunologically-based treatments of the present invention, as detailed

25        below.

Gelsolin is a calcium binding protein that binds to fragments and actin filaments. Mutations at position 187 (e.g., Asp  $\rightarrow$  Asn; Asp  $\rightarrow$  Tyr) of the protein result in a form of hereditary systemic amyloidosis, usually found in patients from

Finland, as well as persons of Dutch or Japanese origin. In afflicted individuals, fibrils formed from gelsolin fragments (Agel), usually consist of amino acids 173-243 (68 kDa carboxyterminal fragment) and are deposited in blood vessels and basement membranes, resulting in corneal dystrophy and cranial neuropathy which progresses to peripheral neuropathy, dystrophic skin changes and deposition in other organs. (Kangas, H., *et al.* Human Mol. Genet. 5(9): 1237-1243, 1996).

Other mutated proteins, such as mutant alpha chain of fibrinogen (AfibA) and mutant cystatin C (Acys) also form fibrils and produce characteristic hereditary disorders. AfibA fibrils form deposits characteristic of a nonneuropathic hereditary amyloid with renal disease; Acys deposits are characteristic of a hereditary cerebral amyloid angiopathy reported in Iceland. (Isselbacher, *Ct at.*, Harrison's Principles of Internal Medicine, McGraw-Hill, San Francisco, 1995; Benson, *et al.*, *supra.*). In at least some cases, patients with cerebral amyloid angiopathy (CAA) have been shown to have amyloid fibrils containing a non-mutant form of cystatin C in conjunction with beta protein. (Nagai, A., *et al.* Molec. Chem. Neuropathol. 33: 63-78, 1998).

Certain forms of prion disease are now considered to be heritable, accounting for up to 15% of cases, which were previously thought to be predominantly infectious in nature. (Baldwin, *et al.*, in Research Advances in *Alzheimer's Disease and Related Disorders*, John Wiley and Sons, New York, 1995). In such prion disorders, patients develop plaques composed of abnormal isoforms of the normal prion protein (PrP<sup>Sc</sup>). A predominant mutant isoform, PrP<sup>Sc</sup>, also referred to as AScr, differs from the normal cellular protein in its resistance to protease degradation, insolubility after detergent extraction, deposition in secondary lysosomes, post-translational synthesis, and high  $\beta$ -pleated sheet content. Genetic linkage has been established for at least five mutations resulting in Creutzfeldt-Jacob disease (CJD), Gerstmann-Sträussler-Scheinker syndrome (GSS), and fatal familial insomnia (FFI). (Baldwin) Methods for extracting fibril peptides from scrapie fibrils, determining

sequences and making such peptides are known in the art. (e.g., Beekes, M., *et al.* J. Gen. Virol. 76: 2567-76, 1995).

For example, one form of GSS has been linked to a PrP mutation at codon 102, while telencephalic GSS segregates with a mutation at codon 117. Mutations at  
5 codons 198 and 217 result in a form of GSS in which neuritic plaques characteristic of Alzheimer's disease contain PrP instead of A $\beta$  peptide. Certain forms of familial CJD have been associated with mutations at codons 200 and 210; mutations at codons 129 and 178 have been found in both familial CJD and FFI. (Baldwin, *supra*).

#### 10 *Senile Systemic Amyloidosis*

Amyloid deposition, either systemic or focal, increases with age. For example, fibrils of wild type transthyretin (TTR) are commonly found in the heart tissue of elderly individuals. These may be asymptomatic, clinically silent, or may result in heart failure. Asymptomatic fibrillar focal deposits may also occur in the  
15 brain (A $\beta$ ), corpora amylacea of the prostate ( $\beta_2$  microglobulin), joints and seminal vesicles.

#### *Cerebral Amyloidosis*

Local deposition of amyloid is common in the brain, particularly in elderly individuals. The most frequent type of amyloid in the brain is composed primarily of  
20 A $\beta$  peptide fibrils, resulting in dementia or sporadic (non-hereditary) Alzheimer's disease. In fact, the incidence of sporadic Alzheimer's disease greatly exceeds forms shown to be hereditary. Fibril peptides forming these plaques are very similar to those described above, with reference to hereditary forms of Alzheimer's disease (AD).

#### 25 *Dialysis-related Amyloidosis*



Plaques composed of  $\beta_2$  microglobulin ( $\beta_2$ M) fibrils commonly develop in patients receiving long term hemodialysis or peritoneal dialysis.  $\beta_2$  microglobulin is a 11.8 kilodalton polypeptide and is the light chain of Class I MHC antigens, which are present on all nucleated cells. Under normal circumstances, it is continuously  
5 shed from cell membranes and is normally filtered by the kidney. Failure of clearance, such as in the case of impaired renal function, leads to deposition in the kidney and other sites (primarily in collagen-rich tissues of the joints). Unlike other fibril proteins,  $\beta_2$ M molecules are generally present in unfragmented form in the fibrils. (Benson, *supra*).

#### 10 *Hormone-derived Amyloidoses*

Endocrine organs may harbor amyloid deposits, particularly in aged individuals. Hormone-secreting tumors may also contain hormone-derived amyloid plaques, the fibrils of which are made up of polypeptide hormones such as calcitonin (medullary carcinoma of the thyroid), islet amyloid polypeptide (amylin; occurring  
15 in most patients with Type II diabetes), and atrial natriuretic peptide (isolated atrial amyloidosis). Sequences and structures of these proteins are well known in the art.

#### *Miscellaneous Amyloidoses*

There are a variety of other forms of amyloid disease that are normally manifest as localized deposits of amyloid. In general, these diseases are probably the  
20 result of the localized production and/or lack of catabolism of specific fibril precursors or a predisposition of a particular tissue (such as the joint) for fibril deposition. Examples of such idiopathic deposition include nodular AL amyloid, cutaneous amyloid, endocrine amyloid, and tumor-related amyloid.

#### **Pharmaceutical Compositions**

25 The pharmaceutical compositions of the present invention are directed to vaccines prepared from fibril peptides that have at least 50% of their amino acid

residues in the dextro form (D-isomers) . Preferably, the vaccines are prepared from all D-A $\beta$  (10-21), D-A $\beta$  (13-21), D-A $\beta$  (25-35), D-A $\beta$  (16-21), D-A $\beta$  (10-16), D-A $\beta$  (1-40), D-A $\beta$  (1-42), D-A $\beta$  (10-22), D-A $\beta$  (13-22), or D-A $\beta$  (16-22), or the N – or C-terminal region of D-A $\beta$ (1-42). The vaccines are believed to elicit an immune response in the host or to produce antibodies that recognize the naturally occurring target. As used herein, “all-D” includes peptides having at least 50% D-configuration amino acids. Preferably, “all-D” also includes peptides having greater than or equal to 50%; 55%; 60%; 65%; 70%; 75%; 80%; 85%; 90%; 95% or 100% D-configuration amino acids.

10 The vaccine according to the present invention is able to prevent the development of brain amyloidosis through two possible scenarios: 1) the effect of anti-A $\beta$  antibodies at the site of amyloid deposition, and 2) the systemic effect of the high circulatory anti-A $\beta$  level on the plasmatic A $\beta$  concentrations.

Specifically, elevated plasma anti-A $\beta$  antibody levels may act systemically by decreasing normal A $\beta$  plasma levels, thereby creating a systemic imbalance in the normal A $\beta$  levels. Such an imbalance could lead to activation of the mechanism responsible for clearing A $\beta$  from the brain and into the periphery, in order to re-establish the normal balance between brain and plasma A $\beta$  levels.

Accordingly, this possibility could be exploited by determining the effect of active or passive immunization on plasma and brain A $\beta$ 40 levels at different timepoints following such immunization. A $\beta$ -immunization can also exert a systemic protective effect against the development of brain amyloidosis. The ratio of A $\beta$  levels in plasma and brain should remain constant in immunized transgenic animals, while it should decrease in control animals. Additionally, B-cell or bone marrow cell transfer from immunized to naïve transgenic animals should have the same effect as passive immunization using anti-A $\beta$  antibodies.

Furthermore, the vaccine of the present invention does not present the drawbacks of using "self" proteins and does not need to be aggregated to induce an immune response. For example, the antibodies raised against the all-D-A $\beta$ (16-21) peptide can be expected to recognize the all-L-A $\beta$ (16-21) peptide sequence.

- 5    Pharmaceutical compositions of the present invention may include, in addition to the immunogenic peptide(s), an effective amount of an adjuvant and/or an excipient. Pharmaceutically effective and useful adjuvants and excipients are well known in the art, and are described in more detail below.

- 10    According to the present invention, compositions capable of eliciting or providing an immune response directed to certain components of amyloid plaques are effective to treat or prevent development of amyloid diseases. In particular, according to the invention provided herein, it is possible to prevent progression of, ameliorate the symptoms of, and/or reduce the amyloid deposition process in afflicted individuals, when an immunostimulatory dose of an anti-amyloid peptide, 15    or corresponding anti-amyloid immune peptide, is administered to the patient. This section describes exemplary anti-amyloid peptides that produce active, as well as passive, immune responses to amyloid protein and provides exemplary data showing the effect of treatment using such compositions on amyloid brain concentration.

*Anti-Amyloid Peptides: Antibodies, Analogs and Fragments of Amyloid Proteins*

- 20    Generally, anti-amyloid peptides of the invention are composed of a specific plaque component, preferably an amyloid or amyloidogenic component, which is usually a characteristic protein, peptide, or fragment thereof. The human forms of A $\beta$  are referred to as A $\beta$ 39, A $\beta$ 40, A $\beta$ 41, A $\beta$ 42 and A $\beta$ 43. The sequences of these peptides and their relationship to the APP precursor are illustrated by Fig. 1 of Hardy 25    et al., TINS 20, 155-158 (1997). For example, A $\beta$ 42 has the sequence:

H<sub>2</sub>N-Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-  
His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-

Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met-Val-Gly-Gly-Val-  
Val-Ile-Ala-OH (SEQ ID NO:1)

A $\beta$ 41, A $\beta$ 40 and A $\beta$ 39 differ from A $\beta$ 42 by the omission of Ala, Ala-Ile and  
Ala-Ile-Val respectively from the C-terminal end. A $\beta$ 43 differs from A $\beta$ 42 by the  
5 presence of a threonine residue at the C-terminus.

Immunogenic fragments of A $\beta$  are advantageous relative to the intact  
molecule in the present methods for several reasons. First, because only certain  
epitopes within A $\beta$  induce a useful immunogenic response for treatment of  
Alzheimer's disease, an equal dosage of mass of a fragment containing such epitopes  
10 provides a greater molar concentration of the useful immunogenic epitopes than a  
dosage of intact A $\beta$ . Second, certain immunogenic fragments of A $\beta$  generate an  
immunogenic response against amyloid protein without generating a significant  
immunogenic response against APP protein from which A $\beta$  derives. Third,  
fragments of A $\beta$  are simpler to manufacture than intact A $\beta$  due to their shorter size.  
15 Fourth, fragments of A $\beta$  do not aggregate in the same manner as intact A $\beta$ ,  
simplifying preparation of pharmaceutical compositions and administration thereof.  
Fifth, full-length A $\beta$  is toxic to cells, but fragments are not, and A $\beta$  aggregates could  
act as seeds to accelerate plaque deposition, but non-aggregated fragments would  
not. Sixth, A $\beta$  has the unusual property that it can fix and activate both classical and  
20 alternate complement cascades. In particular, it binds to C1q and ultimately to C3bi.  
This association facilitates binding to macrophages leading to activation of B cells.  
In addition, C3bi breaks down further and then binds to CR2 on B cells in a T cell  
dependent manner leading to a 10,000 fold increase in activation of these cells. This  
mechanism causes A $\beta$  to generate an immune response in excess of that of other  
25 antigens (Bradt et al., *Complement-dependent Proinflammatory Properties of the  
Alzheimer's Disease  $\beta$ -Peptide*, J. Exp. Med. Vol.188, pp. 431-438 (1998);  
Thornton et al., Clin. Exp. Immunol. Vol. 104, pp. 531-7 (1996); Jacquier-Sarlin et  
al., Immunology vol. 84, pp. 164-70 (1995).

Some immunogenic fragments of A $\beta$  have a sequence of at least 2, 3, 5, 6, 10 or 20 contiguous amino acids from a natural peptide. Some immunogenic fragments have no more than 10, 9, 8, 7, 5 or 3 contiguous residues from A $\beta$ . Preferred immunogenic fragments include residues 1-42 of A $\beta$ . In some methods, the antibody  
5 specifically binds to an epitope within residues 16-21 of A $\beta$ . In some methods, the antibody specifically binds to an epitope within residues 13-21 of A $\beta$ . In some methods, the antibody specifically binds to an epitope within residues 10-21 of A $\beta$ . In some methods, the antibody specifically binds to an epitope within residues 10-16 of A $\beta$ . In some methods, the antibody specifically binds to an epitope within  
10 residues 25-35 of A $\beta$ . The designation A $\beta$ 16-21 for example, indicates a fragment including residues 16-21 of A $\beta$  and lacking other residues of A $\beta$ . Other less preferred fragments include A $\beta$  1-5, 1-6, 1-7, 1-10, 3-7, 1-3, and 1-4. These fragments require screening for activity in clearing or preventing amyloid deposits as described in the Examples before use. Fragments lacking at least one, and sometimes  
15 at least 5 or 10 N- or C-terminal amino acids present in naturally occurring forms of A $\beta$  are used in some methods. For example, a fragment lacking 5 amino acids from the C-terminal end of A $\beta$ 43 includes the first 38 amino acids from the N-terminal end of A $\beta$ . Unless otherwise indicated, reference to A $\beta$  includes the natural human amino acid sequences indicated above as well as analogs including allelic,  
20 species and induced variants. Analogs typically differ from naturally occurring peptides at one, two or a few positions, often by virtue of conservative substitutions. Analogs typically exhibit at least 50% sequence identity with natural peptides, and preferably 60%, 70%, 80% and most preferably 90% sequence identity. Some analogs also include unnatural amino acids or modifications of N or C terminal  
25 amino acids at one, two or a few positions. For example, the natural aspartic acid residue at position 1 and/or 7 of A $\beta$  can be replaced with iso-aspartic acid. Examples of unnatural amino acids are D-amino acids,  $\alpha$ ,  $\alpha$ -disubstituted amino acids, N-alkyl amino acids, lactic acid, 4-hydroxyproline, gamma-carboxyglutamate,  $\epsilon$ -N,N,N-trimethyllysine,  $\epsilon$ -N-acetyllysine, O-phosphoserine, N-acetylserine, N-  
30 formylmethionine, 3-methylhistidine, 5-hydroxylysine,  $\omega$ -N-methylarginine, and

isoaspartic acid. Fragments and analogs can be screened for prophylactic or therapeutic efficacy in transgenic animal models in comparison with untreated or placebo controls as described below.

A $\beta$ , its fragments and analogs can be synthesized by solid phase peptide  
5 synthesis or recombinant expression, or can be obtained from natural sources. Automatic peptide synthesizers are commercially available from numerous suppliers, such as Applied Biosystems, Foster City, California. Recombinant expression can be in bacteria, such as E. coli, yeast, insect cells or mammalian cells. Procedures for recombinant expression are described by Sambrook et al., *Molecular*  
10 *Cloning: A Laboratory Manual* (C.S.H.P. Press, NY 2d ed., 1989). Some forms of A $\beta$  peptide are also available commercially (e.g., American Peptides Company, Inc., Sunnyvale, CA and California Peptide Research, Inc. Napa, CA).

Therapeutic peptides also include longer polypeptides that include, for example, an active fragment of A $\beta$  peptide, together with other amino acids. Other  
15 amino acids can include those having adjuvant properties or immunostimulant properties and those which serve to increase the stability of the peptide. For example, preferred peptides include fusion proteins comprising a segment of A $\beta$  fused to a heterologous amino acid sequence that induces a helper T-cell response against the heterologous amino acid sequence and thereby a B-cell response against  
20 the A $\beta$  segment. Such polypeptides can be screened for prophylactic or therapeutic efficacy in animal models in comparison with untreated or placebo controls as described below. The A $\beta$  peptide, analog, active fragment or other polypeptide can be administered in associated or multimeric form or in dissociated form. Therapeutic peptides also include multimers of monomeric and oligomeric immunogenic  
25 peptides. More generally, therapeutic peptides for use in the present invention produce or induce an immune response against an amyloid protein. Antibodies may also bind the soluble form in the periphery to act as a sink and drive A $\beta$  from the brain into the periphery, facilitating the clearance of A $\beta$  from the brain by either modulating A $\beta$  equilibrium from the CNS to the periphery or forming a stable

complex with A $\beta$  in the periphery. Such peptides therefore include, but are not limited to, the component itself and variants thereof, analogs and mimetics of the component that induce and/or cross-react with antibodies to the component, as well as antibodies or T-cells that are specifically reactive with the amyloid peptide.

- 5 Induction of an immune response can be active, as when an immunogen is administered to induce antibodies or T-cells reactive with the component in a patient, or passive, as when an antibody is administered that itself binds to the amyloid peptide in the patient. Exemplary peptides for inducing or producing an immune response against amyloid protein are described below.

- 10 One general class of preferred anti-amyloid peptides consists of peptides that are derived from amyloid proteins. As mentioned above, the hallmark of amyloid diseases is the deposition in an organ or organ of amyloid plaques consisting mainly of fibrils, which, in turn, are composed of characteristic amyloid proteins or peptides. According to the present invention, such an amyloid protein or peptide  
15 component is a useful peptide for inducing an anti-amyloid immune response. Table 1 summarizes exemplary fibril-forming proteins that are characteristic of various amyloid diseases. In accordance with this aspect of the present invention, administration to an afflicted or susceptible individual of an immunostimulatory composition which includes the appropriate amyloid protein or peptide, including  
20 homologs or fragments thereof, provides therapy or prophylaxis with respect to the amyloid disease.

- Other formulations for treating hereditary forms of amyloidosis, discussed above, include compositions that produce immune responses against gelsolin fragments for treatment of hereditary systemic amyloidosis, mutant lysozyme protein  
25 (Alys), for treatment of a hereditary neuropathy, mutant alpha chain of fibrinogen (A $\alpha$ 1B) for a non-neuropathic form of amyloidosis manifest as renal disease, and mutant cystatin C (Acys) for treatment of a form of hereditary cerebral angiopathy reported in Iceland. In addition, certain hereditary forms of prion disease (e.g., Creutzfeldt-Jacob disease (CJD), Gerstmann-Sträussler-Scheinker syndrome (GSS),

and fatal familial insomnia (FFI)) are characterized by a mutant isoform of prion protein, PrP<sup>Sc</sup>. This protein can be used in therapeutic compositions for treatment and prevention of deposition of PrP plaques, in accordance with the present invention.

5           As discussed above, amyloid deposition, either systemic or focal, is also associated with aging. It is a further aspect of the present invention that such deposition can be prevented or treated by administering to susceptible individuals compositions consisting of one or more proteins associated with such aging. Thus, plaques composed of ATTR derived from wild type TTR are frequently found in  
10 heart tissue of the elderly. Similarly, certain elderly individuals may develop asymptomatic fibrillar focal deposits of A $\beta$  in their brains; A $\beta$  peptide treatment, as detailed herein may be warranted in such individuals. By way of further example, but not limitation, there are a number of additional, non-hereditary forms of amyloid disease that are candidates for treatment methods of the present invention.  $\beta_2$   
15 microglobulin fibrillar plaques commonly develop in patients receiving long term hemodialysis or peritoneal dialysis. Such patients may be treated with therapeutic compositions directed to  $\beta_2$  microglobulin or, more preferably, immunogenic epitopes thereof, in accordance with the present invention.

Hormone-secreting tumors may also contain hormone-derived amyloid  
20 plaques, the composition of which are generally characteristic of the particular endocrine organ affected. Thus such fibrils may be made up of polypeptide hormones such as calcitonin (medullary carcinoma of the thyroid), islet amyloid polypeptide (occurring in most patients with Type II diabetes), and atrial natriuretic peptide (isolated atrial amyloidosis). Compositions directed at amyloid deposits  
25 which form in the aortic intima in atherosclerosis are also contemplated by the present invention. For example, Westermark, et al. describe a 69 amino acid N-terminal fragment of Apolipoprotein A which forms such plaques (Westermark, *et al.* Am. J. Path. 147: 1186-92, 1995); therapeutic compositions of the present



invention include immunological peptides directed to such a fragment, as well as the fragment itself.

The foregoing discussion has focused on amyloid fibril components that may be used as therapeutic peptides in treating or preventing various forms of amyloid  
5 disease.

The therapeutic peptide can also be an active fragment or analog of a naturally occurring or mutant fibril peptide or protein that contains an epitope that induces a similar protective or therapeutic immune response on administration to a human. Immunogenic fragments typically have a sequence of at least 3, 5, 6, 10 or  
10 20 contiguous amino acids from a natural peptide. Exemplary A $\beta$  peptide immunogenic fragments include A $\beta$  residues 16-21; residues 13-21; residues 10-21; residues 10-16; residues 25-35 residues 10-22; residues 13-22; residues 16-22; and of A $\beta$ . In some methods, the antibody binds to an epitope comprising a free N - or C-terminal residue of A $\beta$ .

15 Fragments lacking at least one, and sometimes at least 5 or 10 N- or C-terminal amino acids present in naturally occurring forms of the fibril component are used in some methods. For example, a fragment lacking 5 amino acids from the C-terminal end of A $\beta$ 43 includes the first 38 amino acids from the N-terminal end of A $\beta$ . Fragments from the N-terminal half of A $\beta$  are preferred in some methods.  
20 Analogs include allelic, species and induced variants. Analogs typically differ from naturally occurring peptides at one or a few positions, often by virtue of conservative substitutions. Analogs typically exhibit at least 80 or 90% sequence identity with natural peptides. Some analogs also include unnatural amino acids or modifications of N or C terminal amino acids. Examples of unnatural amino acids are alpha, alpha-  
25 disubstituted amino acids, N-alkyl amino acids, lactic acid, 4-hydroxyproline, (-carboxyglutamate, (-N,N,N-trimethyllysine, (-N-acetyllysine, O-phosphoserine, N-acetylserine, N-formylmethionine, 3-methylhistidine, 5-hydroxylysine, T-N-methylarginine.

Generally, persons skilled in the art will appreciate that fragments and analogs designed in accordance with this aspect of the invention can be screened for cross-reactivity with the naturally occurring fibril components and/or prophylactic or therapeutic efficacy in transgenic animal models as described below. Such fragments  
5 or analogs may be used in therapeutic compositions of the present invention, if their immunoreactivity and animal model efficacy is roughly equivalent to or greater than the corresponding parameters measured for the amyloid fibril components.

Such peptides, proteins, or fragments, analogs and other amyloidogenic peptides can be synthesized by solid phase peptide synthesis or recombinant  
10 expression, according to standard methods well known in the art, or can be obtained from natural sources. Exemplary fibril compositions, methods of extraction of fibrils, sequences of fibril peptide or protein components are provided by many of the references cited in conjunction with the descriptions of the specific fibril components provided herein. Additionally, other compositions, methods of  
15 extracting and determining sequences are known in the art available to persons desiring to make and use such compositions. Automatic peptide synthesizers may be used to make such compositions and are commercially available from numerous manufacturers, such as Applied Biosystems (Perkin Elmer; Foster City, California), and procedures for preparing synthetic peptides are known in the art. Recombinant  
20 expression can be in bacteria, such as *E. coli*, yeast, insect cells or mammalian cells; alternatively, proteins can be produced using cell free *in vitro* translation systems known in the art. Procedures for recombinant expression are described by Sambrook et al., *Molecular Cloning: A Laboratory Manual* (C.S.H.P. Press, NY 2d ed., 1989). Certain peptides and proteins are also available commercially; for example, some  
25 forms of A $\beta$  peptide are available from suppliers such as American Peptides Company, Inc., Sunnyvale, California, and California Peptide Research, Inc. Napa, California.

Therapeutic peptides may also be composed of longer polypeptides that include, for example, the active peptide amyloid fragment or analog, together with

other amino acids. For example, A $\beta$  peptide can be present as intact APP protein or a segment thereof, such as the C-100 fragment that begins at the N-terminus of A $\beta$  and continues to the end of APP. Such polypeptides can be screened for prophylactic or therapeutic efficacy in animal models as described below. The A $\beta$  peptide, analog, active fragment or other polypeptide can be administered in associated form (i.e., as an amyloid peptide) or in dissociated form, such as oligomeric, monomeric or soluble form. Therapeutic peptides may also include multimers of monomeric and oligomeric immunogenic peptides or conjugates or carrier proteins, and/or, as mentioned above, may be added to other fibril components, in order to provide a broader range of anti-amyloid plaque activity.

In a further variation, an immunogenic peptide, such as a fragment of A $\beta$ , can be presented by a virus or a bacteria as part of an immunogenic composition. A nucleic acid encoding the immunogenic peptide is incorporated into a genome or episome of the virus or bacteria. Optionally, the nucleic acid is incorporated in such a manner that the immunogenic peptide is expressed as a secreted protein or as a fusion protein with an outer surface protein of a virus or a transmembrane protein of a bacteria so that the peptide is displayed. Viruses or bacteria used in such methods should be nonpathogenic or attenuated. Suitable viruses include adenovirus, HSV, Venezuelan equine encephalitis virus and other alpha viruses, vesicular stomatitis virus, and other rhabdo viruses, vaccinia and fowl pox. Suitable bacteria include Salmonella and Shigella. Fusion of an immunogenic peptide to HBsAg of HBV is particularly suitable. Therapeutic peptides also include peptides and other compounds that do not necessarily have a significant amino acid sequence similarity with A $\beta$  but nevertheless serve as mimetics of A $\beta$  and induce a similar immune response. For example, any peptides and proteins forming  $\beta$ -pleated sheets can be screened for suitability. Anti-idiotypic antibodies against monoclonal antibodies to A $\beta$  or other amyloidogenic peptides can also be used. Such anti-Id antibodies mimic the antigen and generate an immune response to it (see *Essential Immunology* (Roit ed., Blackwell Scientific Publications, Palo Alto, 6th ed.), p. 181). Peptides other

than A $\beta$  peptides should induce an immunogenic response against one or more of the preferred segments of A $\beta$  listed above (e.g., 10-16, 10-21, 13-21, 16-21 and 25-35). Preferably, such peptides induce an immunogenic response that is specifically directed to one of these segments without being directed to other segments of A $\beta$ .

- 5           Random libraries of peptides or other compounds can also be screened for suitability. Combinatorial libraries can be produced for many types of compounds that can be synthesized in a step-by-step fashion. Such compounds include polypeptides, beta-turn mimetics, polysaccharides, phospholipids, hormones, prostaglandins, steroids, aromatic compounds, heterocyclic compounds,
- 10    benzodiazepines, oligomeric N-substituted glycines and oligocarbamates. Large combinatorial libraries of the compounds can be constructed by the encoded synthetic libraries (ESL) method described in Affymax, WO 95/12608, Affymax, WO 93/06121, Columbia University, WO 94/08051, Pharmacoepia, WO 95/35503 and Scripps, WO 95/30642 (each of which is incorporated by reference for all
- 15    purposes). Peptide libraries can also be generated by phage display methods. See, e.g., Devlin, WO 91/1 8980.

- Combinatorial libraries and other compounds are initially screened for suitability by determining their capacity to bind to antibodies or lymphocytes (B or T) known to be specific for A $\beta$  or other amyloidogenic peptides such as ATTR. For
- 20    example, initial screens can be performed with any polyclonal sera or monoclonal antibody to A $\beta$  or any other amyloidogenic peptide of interest. Compounds identified by such screens are then further analyzed for capacity to induce antibodies or reactive lymphocytes to A $\beta$  or other amyloidogenic peptides. For example, multiple dilutions of sera can be tested on microtiter plates that have been precoated
- 25    with amyloid peptide, and a standard ELISA can be performed to test for reactive antibodies to A $\beta$ . Compounds can then be tested for prophylactic and therapeutic efficacy in transgenic animals predisposed to an amyloidogenic disease, as described in the Examples. Such animals include, for example, mice bearing a 717 mutation of APP described by Games et al., supra, and mice bearing a 670/671 Swedish

mutation of APP such as described by McConlogue et al., US 5,612,486 and Hsiao et al., Science 274, 99 (1996); Staufenbiel et al., Proc. Natl. Acad. Sci. USA 94, 13287-13292 (1997); Sturchler-Pierrat et al., Proc. Natl. Acad. Sci. USA 94, 13287-13292 (1997); Borchelt et al., Neuron 19, 939-945 (1997)). The same screening  
5 approach can be used on other potential peptides such as fragments of A $\beta$ , analogs of A $\beta$  and longer peptides including A $\beta$ , described above.

It is appreciated that immunological responses directed at other amyloid plaque components can also be effective in preventing, retarding or reducing plaque deposition in amyloid diseases. Such components may be minor components of  
10 fibrils or associated with fibrils or fibril formation in the plaques, with the caveat that components that are ubiquitous throughout the body, or relatively non-specific to the amyloid deposit, are generally less suitable for use as therapeutic targets.

It is therefore a further discovery of the present invention that peptides that induce an immune response to specific plaque components are useful in treating or  
15 preventing progression of amyloid diseases. This section provides background on several exemplary amyloid plaque-associated molecules. Induction of an immune response against any of these molecules, alone or in combination with immunogenic therapeutic compositions against the fibril components described above or against any of the other non-fibril forming components described below, provides an  
20 additional anti-amyloid treatment regimen, in accordance with the present invention. Also forming part of the present invention are passive immunization regimens based on such plaque components, as described herein.

By way of example, synuclein is a protein that is structurally similar to apolipoproteins but is found in neuronal cytosol, particularly in the vicinity of  
25 presynaptic terminals. There are at least three forms of the protein, termed alpha, beta and gamma synuclein. Recently, it has been shown that alpha and  $\beta$  synuclein are involved in nucleation of amyloid deposits in certain amyloid diseases, particularly Alzheimer's disease. (Clayton, D.F., *et al.*, *TINS* 21(6): 249-255, 1998).

More specifically, a fragment of the NAC domain of alpha and  $\beta$  synuclein (residues 61-95) has been isolated from amyloid plaques in Alzheimer's patients; in fact this fragment comprises about 10% of the plaque that remains insoluble after solubilization with sodium dodecyl sulfate (SDS). (George, J.M., *et al.* Neurosci. News 1: 12-17, 1995). Further, both the full length alpha synuclein and the NAC fragment thereof have been reported to accelerate the aggregation of  $\beta$ -amyloid peptide into insoluble amyloid *in vitro*. (Clayton, *supra*).

Additional components associated with amyloid plaques include non-peptide components. For example, perlecan and perlecan-derived glycosaminoglycans are large heparin sulfate proteoglycans that are present in  $A\beta$ -containing amyloid plaques of Alzheimer's disease and other CNS and systemic amyloidoses, including amylin plaques associated with diabetes. These compounds have been shown to enhance  $A\beta$  fibril formation. Both the core protein and glycosaminoglycan chains of perlecan have been shown to participate in binding to  $A\beta$ . Additional glycosaminoglycans, specifically, dermatan sulfate, chondroitin-4-sulfate, and pentosan polysulfate, are commonly found in amyloid plaques of various types and have also been shown to enhance fibril formation. Dextran sulfate also has this property. This enhancement is significantly reduced when the molecules are de-sulfated. Immunogenic therapeutics directed against the sulfated forms of glycosaminoglycans, including the specific glycosaminoglycans themselves, form an additional embodiment of the present invention, either as a primary or secondary treatment. Production of such molecules, as well as appropriate therapeutic compositions containing such molecules, is within the skill of the ordinary practitioner in the art.

## **25 Immunization Procedures**

The elicited antibodies present in the host having received the vaccine of the present invention bind at the  $A\beta$ (16-21) epitope or other epitopes such as  $A\beta$ (10-21),  $A\beta$ (13-21).  $A\beta$  (10-22),  $A\beta$  (13-22),  $A\beta$  (16-22) and the N- or C-terminal region of

A $\beta$  and have the ability to prevent or reverse amyloidogenesis. The vaccine of the present invention causes the generation of effective anti-amyloidogenic antibodies in the vaccinated host.

A suggested immunization procedure is as follows:

- 5 a) prepare a vaccine from an all-D peptide having a sequence substantially the same as that of a naturally occurring  $\beta$  amyloid peptide, namely A $\beta$  (all-L). The all-D peptide includes a full length A $\beta$  (1-42, all-D), a peptide derived from an immunogenic fragment of A $\beta$  (1-42, all-D), and a related peptidomimetic;
- 10 b) immunize a host with the vaccine to generate an antibody in the host with a binding site capable of preventing fibrillogenesis, associated cellular toxicity and neurodegeneration.

Suitable pharmaceutically acceptable carriers include, without limitation, any non-immunogenic pharmaceutical adjuvants suitable for oral, parenteral, nasal,  
15 mucosal, transdermal, intravascular (IV), intraarterial (IA), intramuscular (IM), and subcutaneous (SC) administration routes, such as phosphate buffer saline (PBS).

The pharmaceutical carriers may contain a vehicle, which carries antigens to antigen-presenting cells. Examples of vehicles are liposomes, immune-stimulating complexes, microfluidized squalene-in-water emulsions, microspheres which may be  
20 composed of poly(lactic/glycolic) acid (PLGA). Particulates of defined dimensions (<5 micron) include, without limitation, oil-in-water microemulsion (MF59) and polymeric microparticles.

The carriers of the present invention may also include chemical and genetic adjuvants or immunostimulants to augment immune responses or to increase the  
25 antigenicity of immunogens. These adjuvants or immunostimulants exert their immunomodulatory properties through several mechanisms such as lymphoid cell

recruitment, cytokine induction, and the facilitation of DNA entry into cells. Cytokine adjuvants include, without limitation, granulocyte-macrophage colony-stimulating factor, interleukin-12, GM-CSF, synthetic muramyl dipeptide analog or monophosphoryl lipid A. Other chemical adjuvants or immunostimulants  
5 include, without limitation, lactic acid bacteria, Al(OH)<sub>3</sub>, muramyl dipeptides and saponins. Examples of microbial adjuvants include, without limitation, CpG motifs, Freund's, muramyl dipeptide, LPS derivatives, heat shock protein (HSP), lipid A derivative, polysaccharides, cholera toxin, killed *Bordetella pertussis* and LT (lymphotoxin *E. coli*). Examples of non-microbial adjuvants include, without  
10 limitation, aluminum salt, alum, mineral oil, iscoms, liposomes, virosomes, archaeosomes, transfersomes, niosomes, cochleates, proteosomes, calcium phosphate, DDA (dimethyldioctadecylammonium bromide), cytokines, hormones and C3d. Such adjuvants are well understood in the art.

The peptide may be coupled to a carrier that will modulate the half-life of the  
15 circulating peptide. This will allow control of the period of protection. The peptide-carrier may also be emulsified in an adjuvant and administered by a standard immunization route.

The compositions and methods of the present invention can be administered therapeutically or prophylactically to treat diseases associated with amyloid- $\beta$  fibril  
20 formation, aggregation or deposition. The compositions and methods of the invention may also act to ameliorate the course of an amyloid- $\beta$  related disease using any of the following mechanisms, which are meant to be illustrative and not limiting: by slowing the rate of amyloid- $\beta$  fibril formation or deposition; by lessening the degree of amyloid- $\beta$  deposition; by inhibiting, preventing or reducing  
25 amyloid- $\beta$  fibril formation; by inhibiting neurodegeneration or cellular toxicity induced by amyloid- $\beta$ ; by inhibiting amyloid- $\beta$  induced inflammation; by enhancing the clearance of amyloid- $\beta$  from the brain; or by reducing the levels of the amyloid- $\beta$ 40 or amyloid- $\beta$ 42 peptides in the brain or the plasma.



In a preferred embodiment, the method is used to treat Alzheimer's disease (e.g. sporadic or familial AD). The method can also be used prophylactically or therapeutically to treat other clinical occurrences of amyloid- $\beta$  deposition, such as in Down's syndrome individuals and in patients with Cerebral Amyloid Angiopathy, hereditary cerebral amyloid angiopathy, hereditary cerebral hemorrhage or hemorrhagic stroke.

Additionally, abnormal accumulation of APP ( $\beta$ -amyloid precursor protein) and of amyloid- $\beta$  protein in muscle fibers has been implicated in the pathology of sporadic inclusion body myositis (IBM) (Askanas, V. et al. (1996) *Proc. Natl. Acad. Sci. USA* 93: 1314-1319; Askanas, V. et al. (1995) *Current Opinion in Rheumatology* 7: 486-496). Accordingly, the compounds of the invention can be used prophylactically or therapeutically in the treatment of disorders in which amyloid- $\beta$  protein is abnormally deposited at non-neurological locations, such as treatment of IBM by delivery of the compounds to muscle fibers.

The vaccine of the present invention will, for the most part, be administered parenterally, such as intravascularly (IV), intraarterially (IA), intramuscularly (IM), subcutaneously (SC), transdermally or the like. In some instances, administration may be oral, nasal, rectal, transdermal or aerosol, where the nature of the vaccine allows for transfer to the vascular system. Usually a single injection will be employed although more than one injection may be used, if desired. Typically the primary immunization will be followed by multiple boosts with an interval of a few weeks, using the same antigen or a further modified antigen if desired. The adjuvants or immunostimulants may also be identical or different if desired. The vaccine may be administered by any convenient means, including syringe, trocar, catheter, or the like. Preferably, the administration will be intravascular, where the site of introduction is not critical to this invention, preferably at a site where there is rapid blood flow, e.g., intravenously, in a peripheral or central vein. Other routes

may find use where the administration is coupled with slow release techniques or a protective matrix.

The use of the vaccine of the present invention in preventing and/or treating Alzheimer's disease and other amyloid related diseases can be validated by raising  
 5 antibodies against the corresponding all-D peptide and testing them to see if they can effectively inhibit or prevent the fibrillogenesis of the natural amyloid peptide (all-L).

The compounds used to prepare vaccines in accordance with the present invention have the common structure of Formula I:

10 
$$R'-(P)-R'' \quad (I),$$

wherein

P is an all-D peptide of a fibril or amyloid protein, *e.g.*,  $\beta$  sheet region, GAG-binding site region, A $\beta$  (1-42, all-D), and macrophage adherence region (10-16, all-D), immunogenic fragments thereof,  
 15 immunogenic derivatives thereof, protein conjugates thereof, immunogenic peptides thereof, and immunogenic peptidomimetics thereof;

R' is an N-terminal substituent selected from the group consisting of:

- 20
- hydrogen;
  - lower alkyl groups, *e.g.*, acyclic or cyclic having 1 to 8 carbon atoms, without or with functional groups, *e.g.*, carboxylate, sulfonate and phosphonate;
  - aromatic groups;

- heterocyclic groups; and
- acyl groups, *e.g.*, alkylcarbonyl, arylcarbonyl, sulfonyl and phosphonyl groups; and

5 R'' is a C-terminal substituent, *e.g.*, hydroxy, alkoxy, aryloxy, unsubstituted or substituted amino groups.

R' and R'' may be identical or different; the alkyl or aryl group of R' and R'' may further be substituted with organic functionalities selected from the group of halides (F, Cl, Br, and I), hydroxyl, alkoxyl, aryloxy, hydroxycarbonyl, alkoxycarbonyl, aryloxycarbonyl, carbamyl, unsubstituted or substituted amino, sulfo or alkyloxysulfonyl, phosphono or alkoxyphosphonyl, and the like.

The term “alkyl” includes saturated aliphatic groups, including straight-chain alkyl groups (*e.g.*, methyl, ethyl, propyl, butyl, pentyl, hexyl, heptyl, and octyl), branched-chain alkyl groups (*e.g.*, isopropyl, tert-butyl, isobutyl), cycloalkyl (*e.g.*, alicyclic) groups (*e.g.*, cyclopropyl, cyclopentyl, cyclohexyl, cycloheptyl, cyclooctyl), alkyl substituted cycloalkyl groups, and cycloalkyl substituted alkyl groups. “Alkyl” further includes alkyl groups which have oxygen, nitrogen, sulfur or phosphorous atoms replacing one or more hydrocarbon backbone carbon atoms. In certain embodiments, a straight chain or branched chain alkyl has six or fewer carbon atoms in its backbone (*e.g.*, C<sub>1</sub>-C<sub>6</sub> for straight chain, C<sub>3</sub>-C<sub>6</sub> for branched chain), and more preferably four or fewer. Likewise, preferred cycloalkyls have from three to eight carbon atoms in their ring structure, and more preferably have five or six carbons in the ring structure. “C<sub>1</sub>-C<sub>6</sub>” includes alkyl groups containing one to six carbon atoms.

The term “alkyl” also includes both “unsubstituted alkyls” and “substituted alkyls”, the latter of which refers to alkyl moieties having substituents replacing a hydrogen on one or more carbons of the hydrocarbon backbone. Such substituents can include, for example, alkyl, alkenyl, alkynyl, halogen, hydroxyl, alkylcarbonyloxy, arylcarbonyloxy, alkoxy carbonyloxy, aryloxy carbonyloxy,

carboxylate, alkylcarbonyl, arylcarbonyl, alkoxycarbonyl, aminocarbonyl, alkylaminocarbonyl, dialkylaminocarbonyl, alkylthiocarbonyl, alkoxyl, phosphate, phosphonato, phosphinato, cyano, amino (including alkylamino, dialkylamino, arylamino, diarylamino, and alkylarylamino), acylamino (including

5 alkylcarbonylamino, arylcarbonylamino, carbamoyl and ureido), amidino, imino, sulfhydryl, alkylthio, arylthio, thiocarboxylate, sulfates, alkylsulfinyl, sulfonato, sulfamoyl, sulfonamido, nitro, trifluoromethyl, cyano, azido, heterocyclyl, alkylaryl, or an aromatic or heteroaromatic moiety. Cycloalkyls can be further substituted, *e.g.*, with the substituents described above. An "alkylaryl" or an "aralkyl" moiety is

10 an alkyl substituted with an aryl (*e.g.*, phenylmethyl (benzyl)). "Alkyl" also includes the side chains of natural and unnatural amino acids.

As used herein, "acyl groups" include compounds and moieties which contain the acyl radical ( $\text{CH}_3\text{CO}-$ ) or a carbonyl group. "Substituted acyl" includes acyl groups where one or more of the hydrogen atoms are replaced by for example,

15 alkyl groups, alkynyl groups, halogens, hydroxyl, alkylcarbonyloxy, arylcarbonyloxy, alkoxycarbonyloxy, aryloxy carbonyloxy, carboxylate, alkylcarbonyl, arylcarbonyl, alkoxycarbonyl, aminocarbonyl, alkylaminocarbonyl, dialkylaminocarbonyl, alkylthiocarbonyl, alkoxyl, phosphate, phosphonato, phosphinato, phosphonyl, cyano, amino (including alkylamino, dialkylamino, arylamino, diarylamino, and alkylarylamino), acylamino (including

20 alkylcarbonylamino, arylcarbonylamino, carbamoyl and ureido), amidino, imino, sulfhydryl, alkylthio, arylthio, thiocarboxylate, sulfates, alkylsulfinyl, sulfonato, sulfamoyl, sulfonamido, sulfonyl nitro, trifluoromethyl, cyano, azido, heterocyclyl, alkylaryl, or an aromatic or heteroaromatic moiety, *i.e.* arylcarbonyl.

25 The term "heterocyclic group" includes closed ring structures, *e.g.*, 3- to 10-; or 4- to 7-membered rings, which include one or more heteroatoms. Heterocyclyl groups can be saturated or unsaturated and include pyrrolidine, oxolane, thiolane, piperidine, piperazine, morpholine, lactones, lactams such as azetidinones and pyrrolidinones, sultams, sultones, and the like. The heterocyclic ring can be

substituted at one or more positions with such substituents as described above, as for example, halogen, hydroxyl, alkylcarbonyloxy, arylcarbonyloxy, alkoxycarbonyloxy, aryloxycarbonyloxy, carboxylate, alkylcarbonyl, alkoxycarbonyl, aminocarbonyl, alkylthiocarbonyl, alkoxyl, phosphate, phosphonato, phosphinato, cyano, amino  
5 (including alkyl amino, dialkylamino, arylamino, diarylamino, and alkylarylamino), acylamino (including alkylcarbonylamino, arylcarbonylamino, carbamoyl and ureido), amidino, imino, sulfhydryl, alkylthio, arylthio, thiocarboxylate, sulfates, sulfonato, sulfamoyl, sulfonamido, nitro, trifluoromethyl, cyano, azido, heterocyclyl, or an aromatic or heteroaromatic moiety.

10 As used herein, the term "aromatic groups" is intended to encompass "aryl" substituents. "Aryl" includes groups with aromaticity, including 5- and 6-membered single-ring aromatic groups that may include from zero to four heteroatoms, as well as polycyclic systems with at least one aromatic ring. Examples of aryl groups include benzene, phenyl, pyrrole, furan, thiophene, thiazole, isothiazole, imidazole,  
15 triazole, tetrazole, pyrazole, oxazole, isooxazole, pyridine, pyrazine, pyridazine, and pyrimidine, and the like. Furthermore, the term "aryl" includes polycyclic aryl groups, *e.g.*, tricyclic, bicyclic, *e.g.*, naphthalene, benzoxazole, benzodioxazole, benzothiazole, benzoimidazole, benzothiophene, methylenedioxyphenyl, quinoline, isoquinoline, naphthridine, indole, benzofuran, purine, benzofuran, deazapurine, or  
20 indolizine. Those aryl groups having heteroatoms in the ring structure may also be referred to as "aryl heterocycles", "heterocycles," "heteroaryls" or "heteroaromatics". The aromatic ring can be substituted at one or more ring positions with such substituents as described above, as for example, halogen, hydroxyl, alkoxy, alkylcarbonyloxy, arylcarbonyloxy, alkoxycarbonyloxy,  
25 aryloxycarbonyloxy, carboxylate, alkylcarbonyl, alkylaminocarbonyl, aralkylaminocarbonyl, alkenylaminocarbonyl, alkylcarbonyl, arylcarbonyl, aralkylcarbonyl, alkenylcarbonyl, alkoxycarbonyl, aminocarbonyl, alkylthiocarbonyl, phosphate, phosphonato, phosphinato, cyano, amino (including alkylamino, dialkylamino, arylamino, diarylamino, and alkylarylamino), acylamino (including

alkylcarbonylamino, arylcarbonylamino, carbamoyl and ureido), amidino, imino, sulfhydryl, alkylthio, arylthio, thiocarboxylate, sulfates, alkylsulfinyl, sulfonato, sulfamoyl, sulfonamido, nitro, trifluoromethyl, cyano, azido, heterocyclyl, alkylaryl, or an aromatic or heteroaromatic moiety. Aryl groups can also be fused or bridged with alicyclic or heterocyclic rings which are not aromatic so as to form a multicyclic system (e.g., tetralin, methylenedioxyphenyl).

Where a functional group is an acid, its pharmaceutically acceptable salt or ester is in the scope of this invention. Where a functional group is a base, its pharmaceutically acceptable salt is in the scope of this invention.

10 In one embodiment, P is a peptide capable of interacting with at least one region of an amyloid protein or fibril.

In another embodiment, the preferred compounds are selected from the full-length peptide, A $\beta$  (1-42, all-D), and its lower homologues consisting of A $\beta$  (1-40, all-D), A $\beta$  (1-35, all-D), A $\beta$  (1-28, all-D), and A $\beta$  (10-21, all-D).

15 In another embodiment, the preferred compounds are selected from a group of short peptides, *e.g.*, A $\beta$  (1-7, all-D), A $\beta$  (10-16, all-D), A $\beta$  (13-21, all-D), A $\beta$  (16-21, all-D), A $\beta$  (36-42, all-D) AB (10-22 all-D), AB (13-22 all-D) or AB (16-22 all D). The peptides can be shortened further by removing one or more residues from either end or both ends.

20 The preferred compounds may also be all-D peptides derived from the peptides above by substitution of one or more residues in the sequence with other amino acid residues or non-amino acid fragments, such as an amino alkanesulfonic acid residue.

25 In a further embodiment, the preferred compounds may be coupled with a carrier that will modulate the biodistribution, immunogenic property and the half-life of the compounds.

The following are exemplary compounds for preparing vaccines for preventing or treating Alzheimer's disease and other amyloid related diseases:

5	SEQ ID NO: 1	A $\beta$ (1-42, all-D) DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMV GGVVIA
	SEQ ID NO: 2	A $\beta$ (1-40, all-D) DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMV GGVV
10	SEQ ID NO: 3	A $\beta$ (1-35, all-D) DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLM
	SEQ ID NO: 4	A $\beta$ (1-28, all-D) DAEFRHDSGYEVHHQKLVFFAEDVGSNK
15	SEQ ID NO: 5	A $\beta$ (1-7, all-D) DAEFRHD
	SEQ ID NO: 6	A $\beta$ (10-16, all-D) YEVHHQK
20	SEQ ID NO: 7	A $\beta$ (16-21, all-D) KLVFFA
	SEQ ID NO: 8	A $\beta$ (10-21, all-D) YEVHHQKLVFFA
25	SEQ ID NO: 9	A $\beta$ (13-21, all-D) HHQKLVFFA
	SEQ ID NO: 10	A $\beta$ (36-42, all-D) VGGVVIA
	SEQ ID NO: 11	Lys-Ile-Val-Phe-Phe-Ala (all-D)

	SEQ ID NO: 12	Lys-Lys-Leu-Val-Phe-Phe-Ala (all-D)
	SEQ ID NO: 13	Lys-Phe-Val-Phe-Phe-Ala (all-D)
	SEQ ID NO: 14	Ala-Phe-Phe-Val-Leu-Lys (all-D)
	SEQ ID NO: 15	Lys-Leu-Val-Phe (all-D)
5	SEQ ID NO: 16	Lys-Ala-Val-Phe-Phe-Ala (all-D)
	SEQ ID NO: 17	Lys-Leu-Val-Phe-Phe (all-D)
	SEQ ID NO: 18	Lys-Leu-Val-Phe-Phe-Ala-NH <sub>2</sub> (all-D)
	SEQ ID NO: 19	Lys-Ile-Val-Phe-Phe-Ala-NH <sub>2</sub> (all-D)
	SEQ ID NO: 20	Lys-Leu-Val-Phe-Phe-Ala-NH <sub>2</sub> (all-D)
10	SEQ ID NO: 21	Lys-Phe-Val-Phe-Phe-Ala-NH <sub>2</sub> (all-D)
	SEQ ID NO: 22	Ala-Phe-Phe-Val-Leu-Lys-NH <sub>2</sub> (all-D)
	SEQ ID NO: 23	Lys-Leu-Val-Phe-NH <sub>2</sub> (all-D)
	SEQ ID NO: 24	Lys-Ala-Val-Phe-Phe-Ala-NH <sub>2</sub> (all-D)
	SEQ ID NO: 25	Lys-Leu-Val-Phe-Phe-NH <sub>2</sub> (all-D)
15	SEQ ID NO: 26	Lys-Val-Val-Phe-Phe-Ala-NH <sub>2</sub> (all-D)
	SEQ ID NO: 27	Lys-Leu-Val-Phe-Phe-Ala-Glu (all-D)
	SEQ ID NO: 28	Lys-Leu-Val-Phe-Phe-Ala-Glu-NH <sub>2</sub> (all-D)
	SEQ ID NO: 29	His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu (all-D)
	SEQ ID NO: 30	Asp-Asp-Asp (all-D)
20	SEQ ID NO: 31	Lys-Val-Asp-Asp-Gln-Asp (all-D)



	SEQ ID NO: 32	His-His-Gln-Lys (all-D)
	SEQ ID NO: 33	Phe-Phe-NH-CH <sub>2</sub> CH <sub>2</sub> SO <sub>3</sub> H (all-D)
	SEQ ID NO: 34	Phe-Phe-NH-CH <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub> SO <sub>3</sub> H (all-D)
	SEQ ID NO: 35	Phe-Phe-NH-CH <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub> SO <sub>3</sub> H (all-D)
5	SEQ ID NO: 36	Phe-Tyr-NH-CH <sub>2</sub> CH <sub>2</sub> SO <sub>3</sub> H (all-D)
	SEQ ID NO: 37	Phe-Tyr-NH-CH <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub> SO <sub>3</sub> H (all-D)
	SEQ ID NO: 38	Phe-Tyr-NH-CH <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub> SO <sub>3</sub> H (all-D)
	SEQ ID NO: 39	HO <sub>3</sub> SCH <sub>2</sub> CH <sub>2</sub> -Phe-Phe (all-D)
	SEQ ID NO: 40	HO <sub>3</sub> SCH <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub> -Phe-Phe (all-D)
10	SEQ ID NO: 41	HO <sub>3</sub> SCH <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub> -Phe-Phe (all-D)
	SEQ ID NO: 42	HO <sub>3</sub> SCH <sub>2</sub> CH <sub>2</sub> -Phe-Tyr (all-D)
	SEQ ID NO: 43	HO <sub>3</sub> SCH <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub> -Phe-Tyr (all-D)
	SEQ ID NO: 44	HO <sub>3</sub> SCH <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub> -Phe-Tyr (all-D)
	SEQ ID NO: 45	HO <sub>3</sub> SCH <sub>2</sub> CH <sub>2</sub> -Leu-Val-Phe-Phe-Ala (all-D)
15	SEQ ID NO: 46	HO <sub>3</sub> SCH <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub> -Leu-Val-Phe-Phe-Ala (all-D)
	SEQ ID NO: 47	HO <sub>3</sub> SCH <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub> -Leu-Val-Phe-Phe-Ala (all-D)
	SEQ ID NO: 48	Leu-Val-Phe-Phe-Ala-NH-CH <sub>2</sub> CH <sub>2</sub> SO <sub>3</sub> H (all-D)
	SEQ ID NO: 49	Leu-Val-Phe-Phe-Ala-NH-CH <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub> SO <sub>3</sub> H (all-D)
	SEQ ID NO: 50	Leu-Val-Phe-Phe-Ala-NH-CH <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub> SO <sub>3</sub> H (all-D)

- SEQ ID NO: 51      Aβ ([L] 10-15 [D] 16-21)  
Y[L]-E[L]-V[L]-H[L]-H[L]-Q[L]-K[D]-L[D]-V[D]-F[D]-  
F[D]-A[D]
- 5      SEQ ID NO: 52      Aβ ([D] 16-21 [L] 22-28)  
K[D]-L[D]-V[D]-F[D]-F[D]-A[D]-E[L]-D[L]-V[L]-G[L]-  
S[L]-N[L]-K[L]
- SEQ ID NO: 53      His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val  
(all-D)
- 10      SEQ ID NO: 54      Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Ser-Asn-Lys  
(all-D)
- SEQ ID NO: 55  
Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-  
Phe-Phe-Ala (all-D)
- 15      The compounds listed above may be modified by removing or inserting one  
or more amino acid residues, or by substituting one or more amino acid residues  
with other amino acid or non-amino acid moieties or fragments. .
- The following are exemplary compounds derived from compound 18 (all-D  
KLVFFA-NH<sub>2</sub>; SEQ ID NO: 18) by substituting one or two amino acid residue(s)  
20      with other amino acids.
- SEQ ID NO: 56      Lys-Leu-Val-Trp-Phe-Ala-NH<sub>2</sub>(all-D)
- SEQ ID NO: 57      Lys-Leu-Val-Phe-Trp-Ala- NH<sub>2</sub> (all-D)
- SEQ ID NO: 58      Lys-Leu-Val-Trp-Trp-Ala- NH<sub>2</sub> (all-D)
- SEQ ID NO: 59      Lys-Leu-Val-Tyr-Phe-Ala- NH<sub>2</sub> (all-D)
- 25      SEQ ID NO: 60      Lys-Leu-Val-Phe-Tyr-Ala- NH<sub>2</sub> (all-D)

	SEQ ID NO: 61	Lys-Leu-Val-Tyr-Tyr-Ala- NH <sub>2</sub> (all-D)
	SEQ ID NO: 62	Lys-Leu-Val-Thi-Phe-Ala- NH <sub>2</sub> (all-D)
	SEQ ID NO: 63	Lys-Leu-Val-Phe-Thi-Ala- NH <sub>2</sub> (all-D)
	SEQ ID NO: 64	Lys-Leu-Val-Thi-Thi-Ala- NH <sub>2</sub> (all-D)
5	SEQ ID NO: 65	Lys-Leu-Val-Cha-Phe-Ala- NH <sub>2</sub> (all-D)
	SEQ ID NO: 66	Lys-Leu-Val-Phe-Cha-Ala- NH <sub>2</sub> (all-D)
	SEQ ID NO: 67	Lys-Leu-Val-Cha-Cha-Ala- NH <sub>2</sub> (all-D)
	SEQ ID NO: 68	Lys-Leu-Val-Pgly-Phe-Ala- NH <sub>2</sub> (all-D)
	SEQ ID NO: 69	Lys-Leu-Val-Phe-Pgly-Ala- NH <sub>2</sub> (all-D)
10	SEQ ID NO: 70	Lys-Leu-Val-Pgly-Pgly-Ala- NH <sub>2</sub> (all-D).

For the above compounds, the terms Thi, Cha and Pgly are intended to mean thienylalanine, cyclohexylalanine and phenylglycine, respectively.

Rabbits were immunized with all-D or all-L KLVFFA. Results of the antibody titers obtained are shown in FIG. 7. As seen in FIG. 7, the vaccine of the present invention causes production of antibodies.

In another experiment, adult TgCRND8 mice were immunized with all-D or all-L KLH-amyloid- $\beta$  fragment ([L]-amyloid- $\beta$  13-21 and [D] amyloid- $\beta$  13-21). Results of the antibody titers is shown in Table 2.

**Table 2**

KLH-amyloid- $\beta$ fragment	Mean Plasma Antibody Titer (inverse) in TgCRND8
[L] amyloid- $\beta$ 13-21	<100
[D] amyloid- $\beta$ 13-21	11,000

The present invention encompasses various types of immune responses triggered using the vaccine of the present invention, *e.g.*, amyloid therapies using the vaccine approach.

5           In accordance with the present invention, there is also provided a vaccine which triggers a preferential TH-2 response or a TH-1 response, according to the type of immunization used. By inducing a TH-2 response, anti-inflammatory cytokine production such as IL-4, IL-10 and TGF- $\beta$ , as well as the production of IgG 1 and IgG 2b antibody classes, are favored. Such type of response would be  
10 preferred, as a major inflammatory response in the brain of the patients with AD would be avoided. On the other hand, with a preferred TH-1 response, a pro-inflammatory response with a production of inflammatory cytokines such as IL-1, IL-6, TNF and IFN gamma would be favored. This type of response would more likely trigger activation of the macrophage population. These macrophages  
15 would then phagocytose any particulate deposits (such as plaques) via a complement-activated process as well as via an antibody-mediated process. This approach would be beneficial to clear already organized senile plaques and prevent the formation of new fibrillary deposits. However this response would also be accompanied by an inflammatory response which would be detrimental to the host.

20           Both approaches (*i.e.* TH-1 and TH-2) are of value. The antigen used could be the peptides which contain regions responsible for cellular adherence, *i.e.*, region 10-16, regions responsible for the GAG binding site, *i.e.*, 13-16, regions responsible for the  $\beta$  sheet, *i.e.* 16-21, or regions specific for the C-terminus, *i.e.* 40-42. These peptides could be presented in such a way that either a preferential TH-1 or TH-2  
25 response is obtained, depending on the type of adjuvant used, or depending on the route of administration of the vaccine. For example, a mucosal immunization via nasal administration is possible, since it is known that such a route of administration would favor a TH-2 response.

The present invention will be more readily understood by referring to the following examples, which are given to illustrate the invention rather than to limit its scope.

### EXAMPLE I

- 5           An *in vitro* validation procedure to test the effectiveness of all-D peptide vaccines derived from fibrillogenic amyloid proteins was performed in rabbits or mice to demonstrate that antibodies can be raised against A $\beta$  16-21 (all-D) (see FIG. 7). The antibodies produced were tested to prove that they effectively prevent the fibrillogenesis of natural A $\beta$  (1-40, all-L) *in vitro*. Standard assays for
- 10   fibrillogenesis were used to evaluate activity, such as those based on Thioflavine T, circular dichroism and solubility.

This approach was also used to establish which areas of the A $\beta$  peptide are most effective when used in the form of all-D peptides to prepare antifibrillogenic vaccines. The experiments were performed as follows:

- 15           a) rabbits or mice were immunized with a series of overlapping all-D peptides generated from the A $\beta$  (1-42) sequence, *e.g.*, A $\beta$  (16-21), A $\beta$  (10-21), A $\beta$  (13-21), etc.
- b) antisera were prepared from the immunized rabbits or mice.
- c) these antisera were tested to see which parts of the A $\beta$  sequence
- 20           produce antisera that most effectively prevent fibrillogenesis in the standard assays for fibrillogenesis mentioned above.

## EXAMPLE II

### Effect of Antibodies Against D- and L-A $\beta$ (16-21) Peptide Vaccine on Fibrillogenesis

A validation procedure to test anti-fibrillogenic activity of antibodies raised  
5 against D- and L- A $\beta$  (16-21) peptide was performed.

Rabbits were immunized with D- or L-A $\beta$  (16-21) peptide. Antibodies raised were tested for their antifibrillogenic activities by ThT assay and by electron microscopy (EM).

Antibodies raised against the D- and L- forms of KLVFFA were capable of  
10 blocking the fibrillogenesis process as seen either by the Thioflavin T assay (ThT) (FIGs. 2 and 3) and by EM (FIGs. 4A to 4C). In the ThT assay, fibril formation is monitored by the increase in fluorescence with time. As seen in the Figures, the antibodies were capable of inhibiting such an increase in fluorescence, proving that these antibodies were inhibiting fibrillogenesis.

15 As can be seen in these figures (FIGs. 2 to 4), antibodies raised against the D-peptide have a better anti-fibrillogenic activity than anti-L antibodies.

These results were also confirmed by EM (FIGs. 4A to 4C) where both anti-D and anti-L KLVFFA peptide blocked the fibril formation when compared to control (FIG. 4A). Moreover, again the anti-D peptide has a greater  
20 anti-fibrillogenic activity (FIG. 4B) than the anti-L peptide (FIG. 4C). This goes along with the ThT assay where the decrease in fluorescence was greater with the anti-D peptide antibody than with the anti-L peptide antibody.

### EXAMPLE III

#### Antibody Binding Assay

Brain sections were stained with antibodies raised against KLVFFA peptide (D and L forms). As seen in FIGs 5A to 5D and 6A to 6D, the antibodies were not  
5 capable of binding to aggregated (ThioS positive) A $\beta$ . It can be seen from both sets of figures, which were stained for both plaques (using ThioS) and A $\beta$  peptide (using the antibodies raised) that the antibodies recognize A $\beta$  at the surface of the cells but are not capable of binding to plaques. These results show that the anti-KLVFFA peptide antibody does not bind to aggregated A $\beta$ . These results clearly prove that  
10 the antibody recognizes only the non-aggregated form and blocks fibrillogenesis. By having such activity, the vaccine of the present invention 1) prevents A $\beta$  from organizing itself into a fibril and 2) prevents an inflammatory response being triggered by such an antibody binding to an insoluble form, since the antibody is not able to bind to aggregated A $\beta$ . Furthermore, by maintaining A $\beta$  in a soluble form,  
15 such antibody may favor the clearance of A $\beta$  from the brain prior to its organization as a fibril. Such antibody would also maintain a beneficial equilibrium between the concentration of A $\beta$  in the brain vs. the plasma.

### EXAMPLE IV

#### 20 Effect of Antibodies Against D- and L- A $\beta$ (13-21-KLH) Peptide Vaccine on Amyloid- $\beta$ Accumulation in the Brain and Plasma

Adult TgCRND8 mice (which carry a transgene expressing the human Amyloid Precursor Protein (hAPP)) were immunized with D or L peptide at 9 weeks of age, and every two weeks thereafter (a total of 5 injections). Animals were  
25 sacrificed 10-14 days after the last boost and the accumulation of amyloid- $\beta$  in the brain and plasma of the mice was determined. Brains were homogenized as follows:

First, brains were frozen and the frozen tissue was weighed. Tissue was then homogenized in 4 volumes of 50 mM Tris-HCl pH 8.0 containing 1x protease inhibitor cocktail (Calbiochem #539131) using a Wheaton Overhead Stirrer, speed 3.6. Samples were vortexed and spun in an Eppendorf centrifuge at 16,000x g for 20 min at 4°C. 150 µL of the supernatant was added to 250 µL 8 M guanidium/50 mM Tris-HCl pH8.0. The mixture was vortexed and stored at -80°C. The pellet was thawed and resuspended by vortexing in 7 volumes of 5 M guanidium/50 mM Tris pH8.0 and stored at -80°C. The supernatant and pellet were thawed and sonicated for 15 min at 80°C in an Elma Ultrasonic bath, then frozen on dry ice for 15 min, then thawed and sonicated in an Elma Ultrasonic bath at 80°C for 10 min. Samples were then spun at 16,000x g for 20 min at 4°C, and the supernatant was kept for amyloid-β quantification. Amyloid-β40 and amyloid-β42 were quantified using a human β Amyloid ELISA kit from Biosource International (catalog #88-344).

Specific plasma antibody titers were determined using a standard ELISA procedure (shown in Table 2, *supra*). The data in table 2 indicates that immunization with the [D] 13-21 peptide was much more effective at inducing an immune response than immunization with the [L] 13-21 peptide.

The results also showed a reduction in both soluble and fibrillar (insoluble) Aβ40 level (FIGs. 8A and 8B), a reduction in both soluble and fibrillar Aβ42 level (FIG. 9) and a reduction in total Aβ level (FIGs. 10A and 10B) in the brain of TgCRND8 mice after immunization with [D]13-21-KLH. The results indicate that immunization with D peptides was effective at reducing Aβ levels. Also, D peptides were much more potent than L peptides, as only immunization with D peptides reduced Aβ levels, and immunization with L peptides did not reduce Aβ levels.



## EXAMPLE V

### Passive Immunization Against Amyloid-beta

5

Humanized antibodies are raised and obtained from transgenic animals such as Xenomouse® (Abgenix, Fremont, CA), by exposing the animal to an antigenic amount of an all-D Amyloid- $\beta$  (10-22). An antigenic amount ranges from 10ng to 10mg of the all-D Amyloid- $\beta$  peptide. The immune response can be enhanced by the use of adjuvants as described, thus permitting lower concentrations of the immunogen to be used. The serum antibody levels are monitored through ELISA or other methods of titering an immune response. The antibodies may be directly recovered from the serum of the animal, or B-cells expressing such antibodies may be recovered from an immunized animal for creation of hybridoma lines. The humanized antibodies are purified from serum, ascites or cell culture medium by Protein G chromatography or similar methods of antibody purification.

The antibodies, raised directly in the second or donor mammal or indirectly by creation of a hybridoma line derived from an immune cell from the donor mammal, can be used to immunize a first or subject mammal suffering from an amyloid related disease or disorder, or to prevent an amyloid related disease or disorder. The first mammal is provided with a therapeutically or prophylactically effective dose of the anti-A $\beta$  antibodies. Antibody fragments or primed immune cells such as B-cells, T-cells, or primed antigen presenting cells can also be obtained from a donor, preferably a human donor and more preferably a syngeneic human donor. A therapeutically effective dose is identified as one which decreases the formation or mass of fibrillar plaques in the first mammal, by at least 5 to 50% or greater, as measured by the techniques and assays described. A 100% or greater reduction in plaque formation or mass is considered a therapeutic endpoint.

Physicians or other medical professionals can monitor dosing regimens to achieve a therapeutic effect or endpoint.

- 5           While the invention has been described in connection with specific  
embodiments thereof, it will be understood that it is capable of further modifications  
and this application is intended to cover any variations, uses, or adaptations of the  
invention following, in general, the principles of the invention and including such  
departures from the present disclosure as come within known or customary practice  
10   within the art to which the invention pertains and as may be applied to the essential  
features hereinbefore set forth, and as follows in the scope of the appended claims.

*What is claimed is:*

1. A method for preventing and/or treating an amyloid-related disease in a subject, comprising: administering to the subject an antigenic amount of an all-D amyloid- $\beta$  peptide, wherein said all-D amyloid- $\beta$  peptide induces an immune response by said subject against said amyloid- $\beta$  peptide, and  
5 wherein said all-D amyloid- $\beta$  peptide has an amino acid sequence that is at least 80% identical to a sequence selected from the group consisting of SEQ ID NOS: 1-70.
- 10 2. The method of claim 1, wherein said all-D peptide interacts with at least one region of an amyloid protein, said region being selected from the group consisting of: an N- or C-terminal region,  $\beta$  sheet region, GAG-binding site region, macrophage adherence region, immunogenic fragments thereof, protein conjugates thereof, immunogenic derivative peptides thereof,  
15 immunogenic peptides thereof, and immunogenic peptidomimetics thereof.
3. The method of claim 2, wherein said all-D peptide further comprises:
  - (a) an N-terminal substituent selected from the group consisting of:  
hydrogen;  
20 lower alkyl group consisting of acyclic or cyclic having 1 to 8 carbon atoms;  
aromatic group;  
heterocyclic group; and  
acyl group; and
  - 25 (b) a C-terminal substituent selected from the group consisting of hydroxy, alkoxy, aryloxy, unsubstituted and substituted amino groups.

4. The method of claim 3, wherein said alkyl or aromatic group is further substituted with a group selected from the group consisting of halide, hydroxyl, alkoxy, aryloxy, hydroxycarbonyl, alkoxycarbonyl, aryloxycarbonyl, carbamyl, unsubstituted amino, substituted amino, sulfo, alkyloxysulfonyl, phosphono and alkoxyphosphonyl groups.
5. The method of claim 1, wherein said all-D peptide further comprises an acid functional group, or a pharmaceutically acceptable salt or ester form thereof.
6. The method of claim 1, wherein said all-D peptide further comprises a base functional group, or a pharmaceutically acceptable salt form thereof.
7. The method of claim 1, wherein said all-D peptide elicits the clearance of soluble amyloid- $\beta$ , thereby preventing or reducing amyloid-induced cellular toxicity, neurodegeneration, or reducing or inhibiting the formation of plaques.
8. A method for preventing and/or treating an amyloid-related disease in a first mammal in need thereof, comprising administering an antigenic amount of an all-D amyloid- $\beta$  peptide to a second mammal, wherein said all-D amyloid- $\beta$  peptide induces an immune response against the amyloid- $\beta$  peptide in said second mammal, recovering the antibodies or immune cells from said second mammal, and providing a therapeutically or prophylactically effective dose of the antibodies or immune cells to said first mammal.
9. The method of claim 8 wherein said all-D amyloid- $\beta$  peptide has an amino acid sequence that is at least 80% identical to a sequence selected from the group consisting of SEQ ID NOS: 1-70.

10. The method of claim 8, wherein said all-D peptide interacts with at least one region of an amyloid protein, said region being selected from the group consisting of: C-terminal region,  $\beta$  sheet region, GAG-binding site region, macrophage adherence region, immunogenic fragments thereof, protein  
5 conjugates thereof, immunogenic derivative peptides thereof, immunogenic peptides thereof, and immunogenic peptidomimetics thereof.
- 10
11. The method of claim 8, wherein said all-D peptide further comprises:
- (a) an N-terminal substituent selected from the group consisting of:
- hydrogen;
- lower alkyl group consisting of acyclic or cyclic having 1 to 8 carbon  
15 atoms;
- aromatic group;
- heterocyclic group; and
- acyl group; and
- (b) a C-terminal substituent selected from the group consisting of  
20 hydroxy, alkoxy, aryloxy, unsubstituted and substituted amino groups.
12. The method of claim 8, wherein said alkyl or aromatic group is further substituted with a group selected from the group consisting of halide,  
25 hydroxyl, alkoxyl, aryloxy, hydroxycarbonyl, alkoxycarbonyl, aryloxy carbonyl, carbamyl, unsubstituted amino, substituted amino, sulfo, alkyloxysulfonyl, phosphono and alkoxyphosphonyl groups.

13. The method of claim 8, wherein said all-D peptide further comprises an acid functional group, or a pharmaceutically acceptable salt or ester form thereof.
14. The method of claim 8, wherein said all-D peptide further comprises a base functional group, or a pharmaceutically acceptable salt form thereof.
15. The method of claim 8, wherein said all-D peptide elicits the clearance of soluble amyloid- $\beta$ , thereby preventing or reducing amyloid-induced cellular toxicity, neurodegeneration, or reducing or inhibiting the formation of plaques.
16. A method for altering serum levels of amyloid- $\beta$  in a mammal comprising administering to the subject an antigenic amount of an all-D amyloid- $\beta$  peptide, wherein said all-D amyloid- $\beta$  peptide induces an immune response by said subject against said amyloid- $\beta$  peptide, and wherein said all-D amyloid- $\beta$  peptide has an amino acid sequence that is at least 80% identical to a sequence selected from the group consisting of SEQ ID NOS: 1-70.
17. The method of claim 16, wherein altering serum levels of amyloid- $\beta$  in a mammal favors the clearance of soluble amyloid- $\beta$  or fibril amyloid- $\beta$  from the mammal.
18. The method of claim 17, wherein the clearance of soluble amyloid- $\beta$  or fibril amyloid- $\beta$  from the mammal thereby reduces or inhibits the formation of plaques in the brain of said mammal.
19. The method of claim 16, wherein said all-D peptide further comprises:

- (a) an N-terminal substituent selected from the group consisting of:  
hydrogen;  
lower alkyl group consisting of acyclic or cyclic having 1 to 8 carbon atoms;  
5 aromatic group;  
heterocyclic group; and  
acyl group; and
- (b) a C-terminal substituent selected from the group consisting of  
hydroxy, alkoxy, aryloxy, unsubstituted and substituted amino  
10 groups.
20. The method of claim 16, wherein said alkyl or aromatic group is further substituted with a group selected from the group consisting of halide, hydroxyl, alkoxyl, aryloxy, hydroxycarbonyl, alkoxycarbonyl,  
15 aryloxycarbonyl, carbamyl, unsubstituted amino, substituted amino, sulfo, alkyloxysulfonyl, phosphono and alkoxyphosphonyl groups.
21. The method of claim 16, wherein said all-D peptide further comprises an acid functional group, or a pharmaceutically acceptable salt or ester form thereof.  
20
22. The method of claim 16, wherein said all-D peptide further comprises a base functional group, or a pharmaceutically acceptable salt form thereof.
23. The method of claim 16, wherein said all-D peptide elicits the clearance of  
25 soluble amyloid- $\beta$ , thereby preventing or reducing amyloid-induced cellular toxicity or neurodegeneration.
24. The method of claim 1, wherein said amyloid-related disease is a disorder

comprising excessive amyloid- $\beta$  peptides, and inducing said immune response by said subject against said amyloid- $\beta$  peptides thereby reduces the excessive levels of said amyloid- $\beta$  peptides, thereby treating or preventing said amyloid-related disease.

5

25. The method of claim 24, wherein said amyloid-related disease is Alzheimer's disease.

10

26. The method of claim 24, wherein said amyloid-related disease is cerebral amyloid angiopathy.

15

27. The method of claim 8, wherein said amyloid-related disease is a disorder comprising excessive amyloid- $\beta$  peptides, and inducing said immune response by said subject against said amyloid- $\beta$  peptides thereby reduces the excessive levels of said amyloid- $\beta$  peptides, thereby treating or preventing said amyloid-related disease.

20

28. The method of claim 27, wherein said amyloid-related disease is Alzheimer's disease.

29. The method of claim 27, wherein said amyloid-related disease is cerebral amyloid angiopathy.

25

30. The method of claim 16, wherein said amyloid-related disease is a disorder comprising excessive amyloid- $\beta$  peptides, and inducing said immune response by said subject against said amyloid- $\beta$  peptides thereby reduces the excessive levels of said amyloid- $\beta$  peptides, thereby treating or preventing said amyloid-related disease.

30

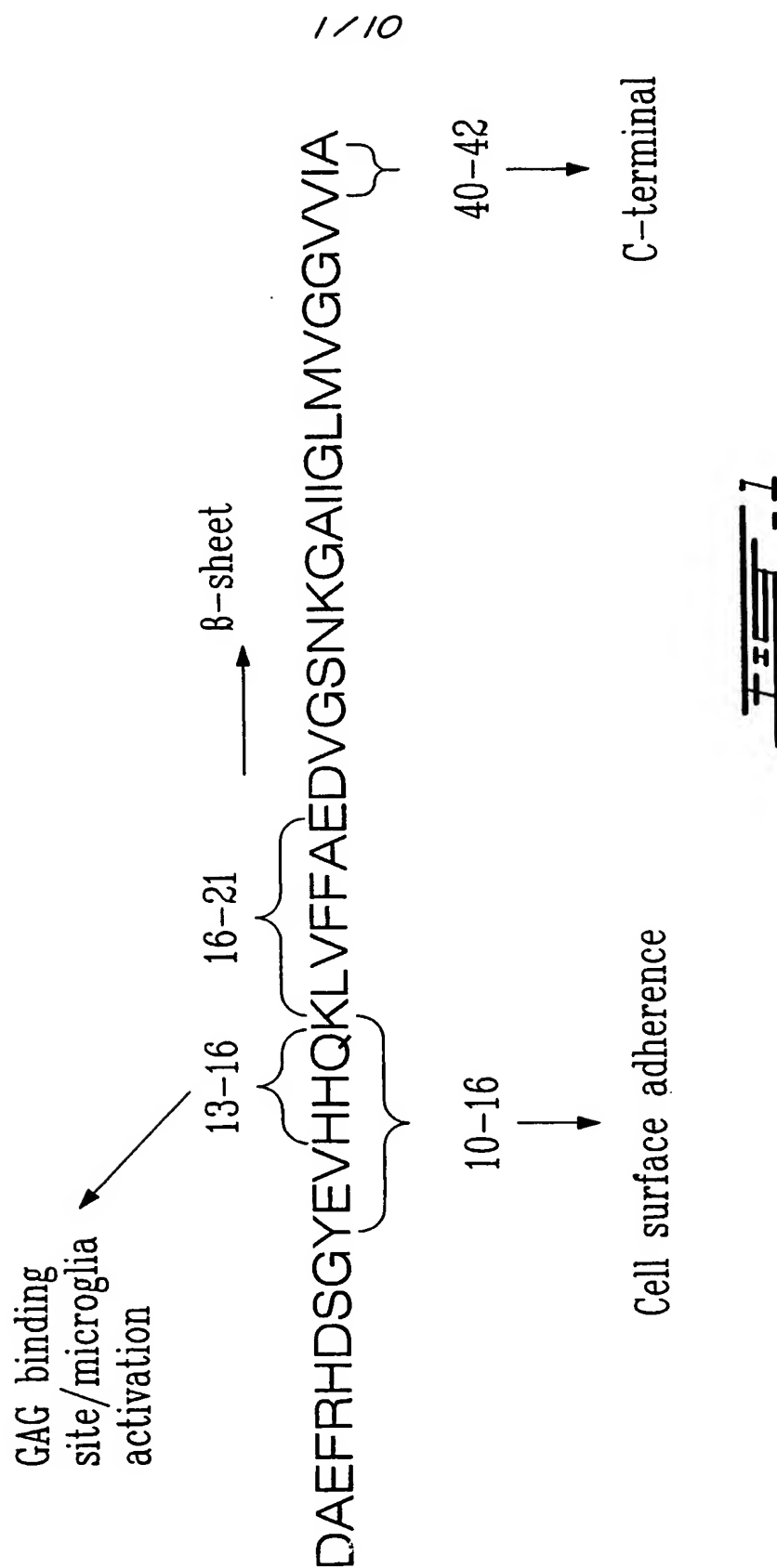
31. The method of claim 30, wherein said amyloid-related disease is Alzheimer's



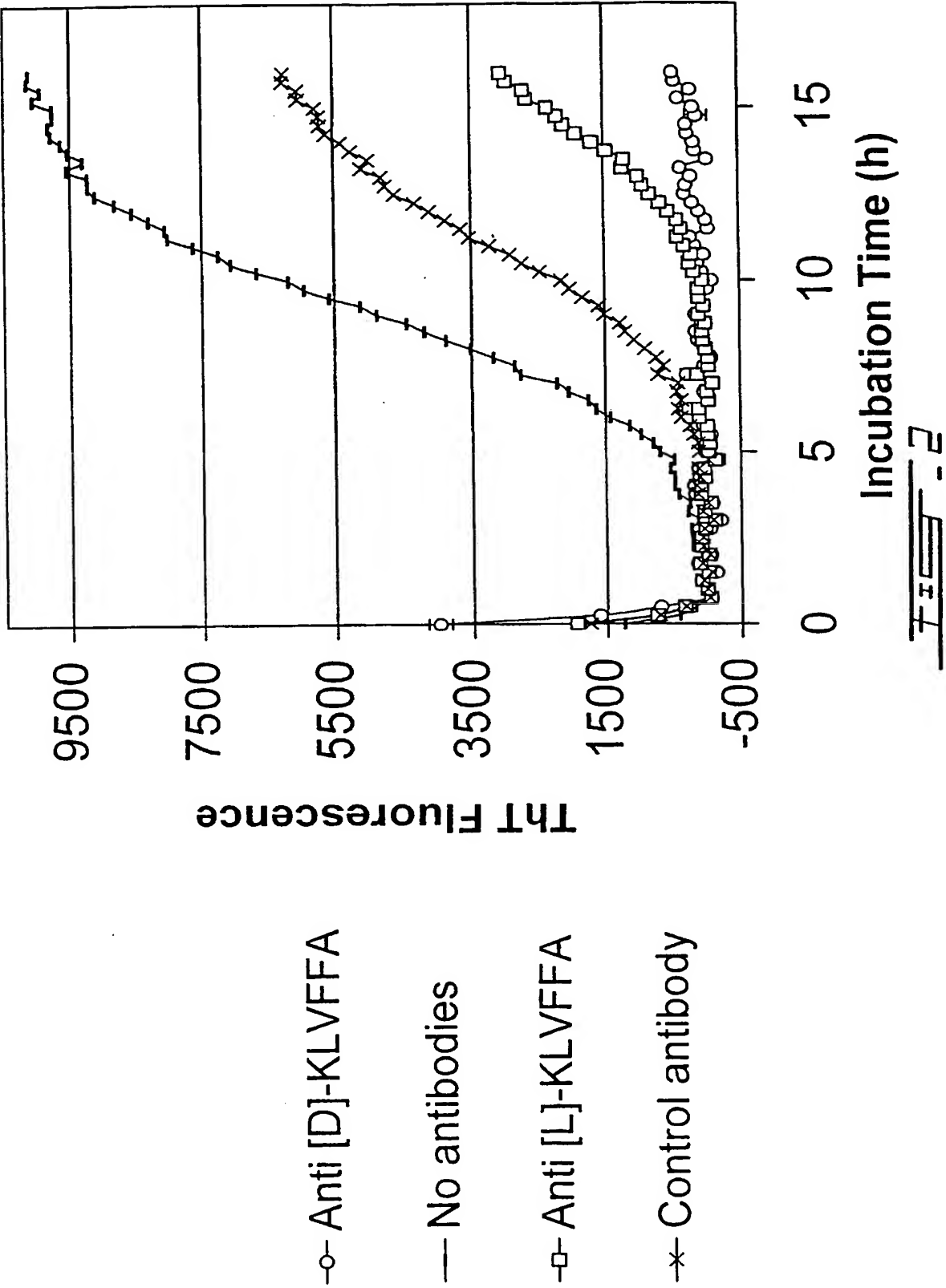
disease.

32. The method of claim 30, wherein said amyloid-related disease is cerebral amyloid angiopathy.

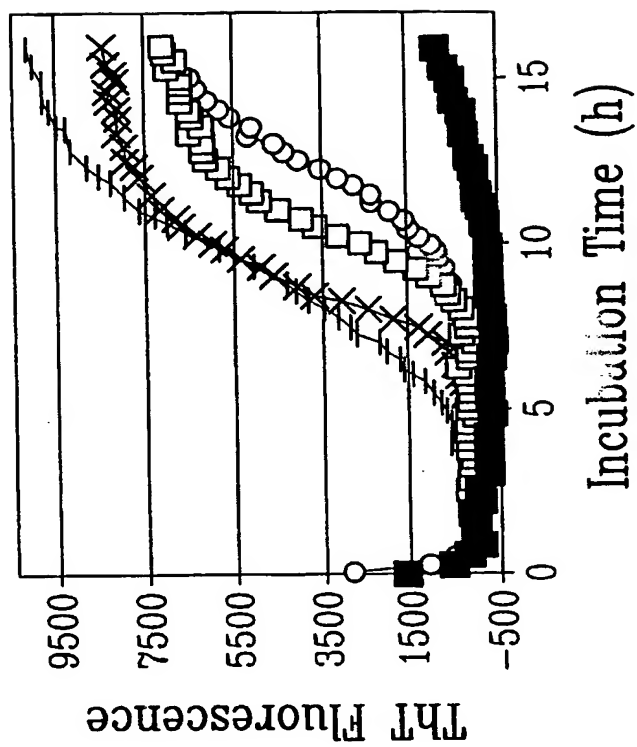
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3/10

Fig. 3

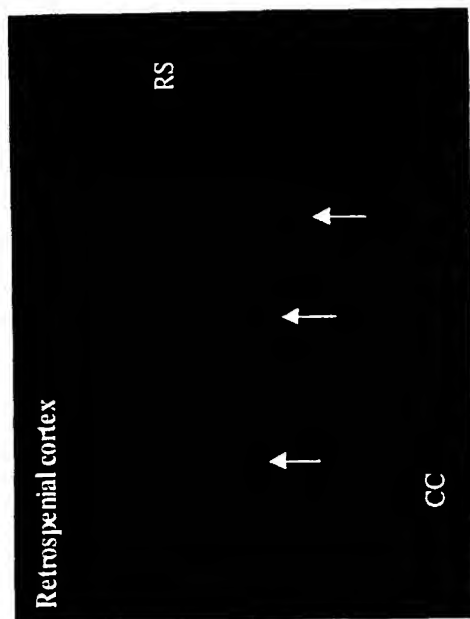
- Anti [D]-KLVFFA
- No antibodies
- × Control antibody
- Anti [L]-KLVFFA
- Anti [D]-KLVFFA binding [L]-KLVFFA

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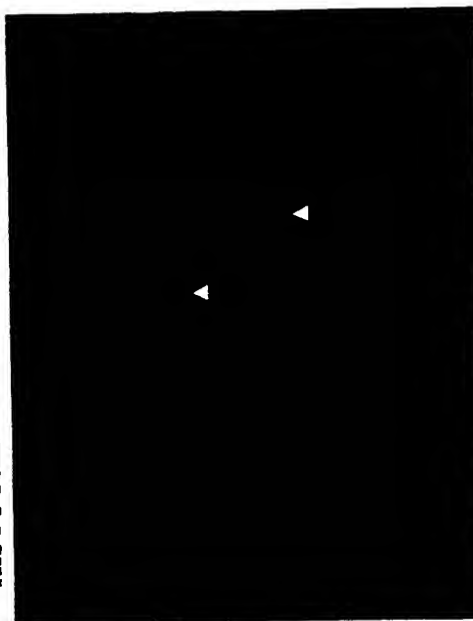
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HISTOCHEMISTRY FOR THIOFLAVIN S



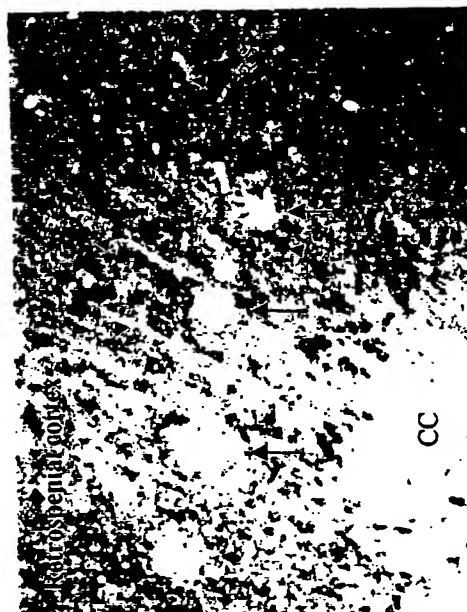
FEF-5B

HISTOCHEMISTRY FOR THIOFLAVIN S



FEF-5D

IMMUNOHISTOCHEMISTRY FOR A 151



FEF-5A

IMMUNOHISTOCHEMISTRY FOR A 151



FEF-5C

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HISTOCHEMISTRY FOR THIOFLAVIN S

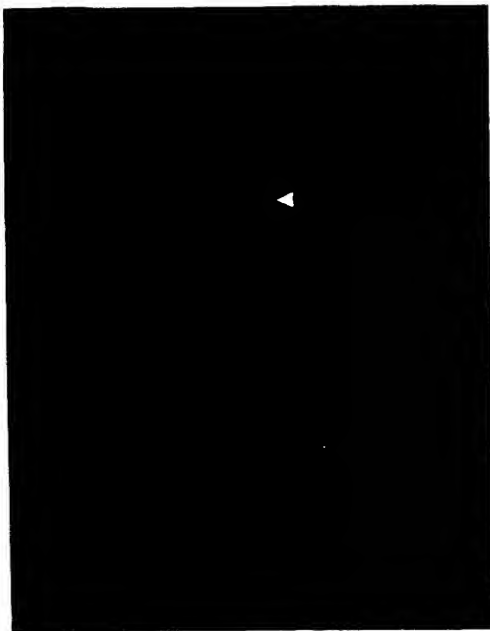


FIG. 6B

HISTOCHEMISTRY FOR THIOFLAVIN S

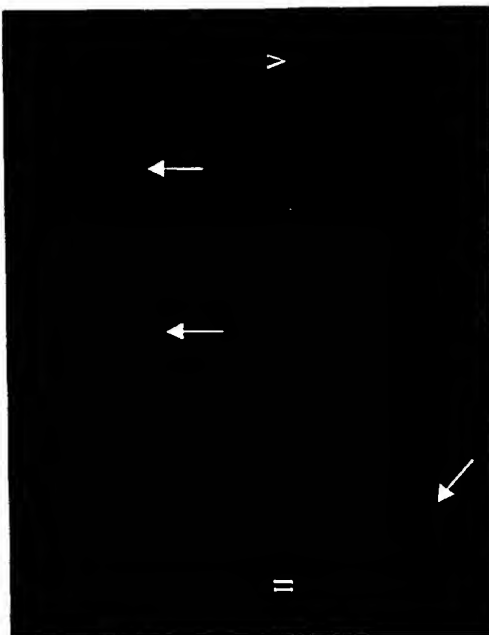


FIG. 6D

IMMUNOHISTOCHEMISTRY FOR C151

Parietal cortex



FIG. 6A

IMMUNOHISTOCHEMISTRY FOR C151

Entorhinal cortex

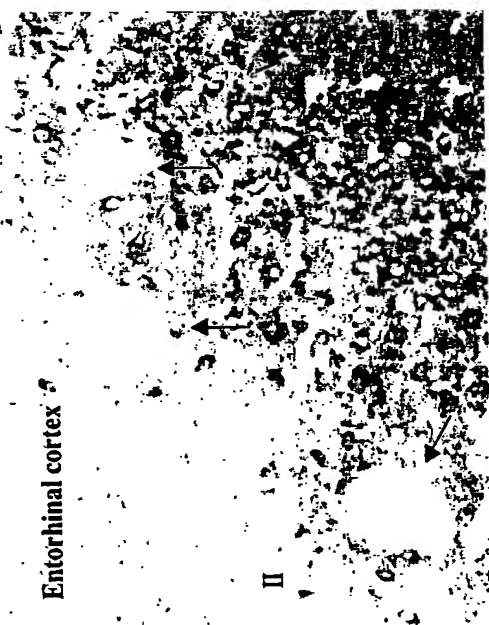
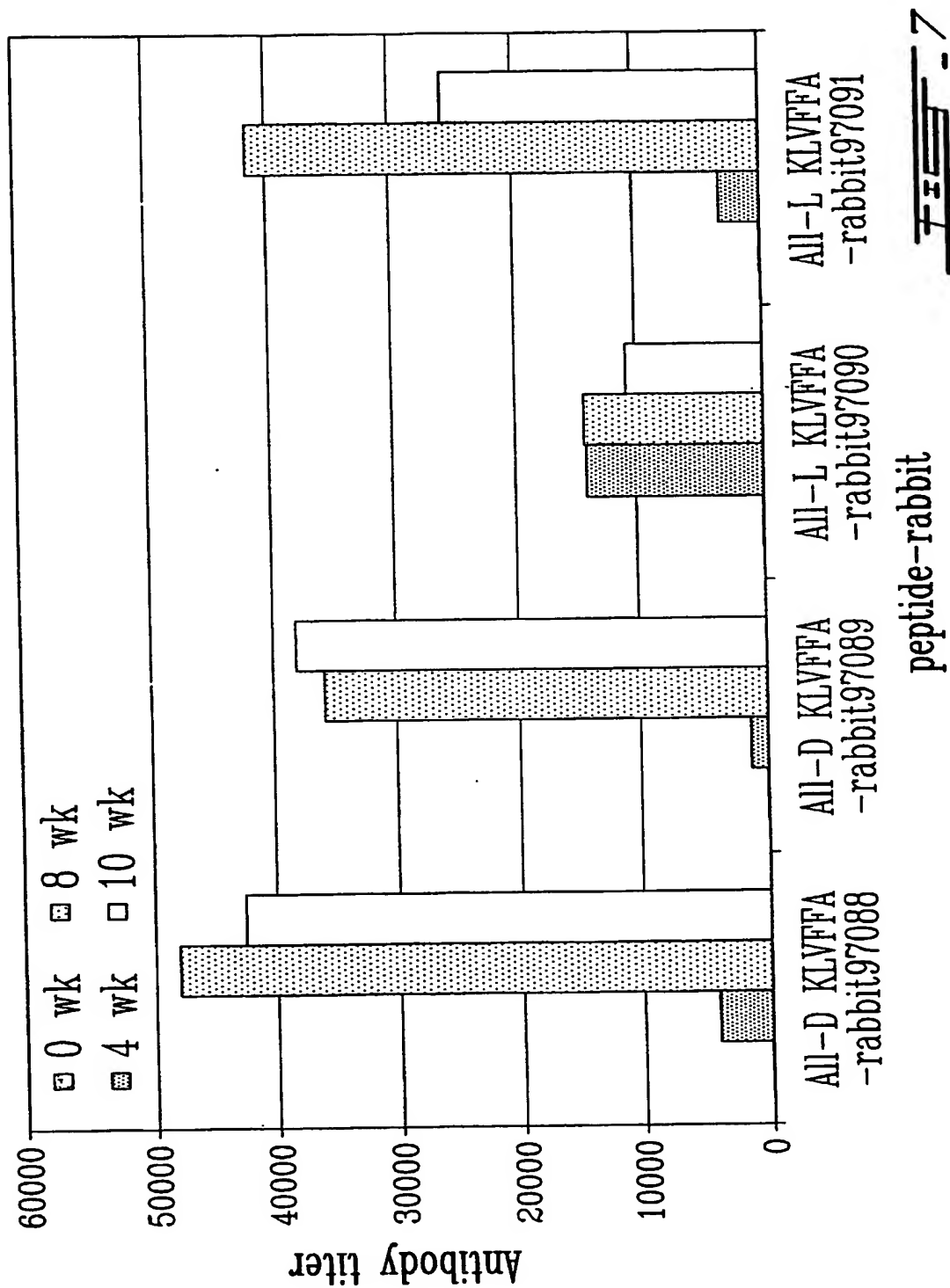


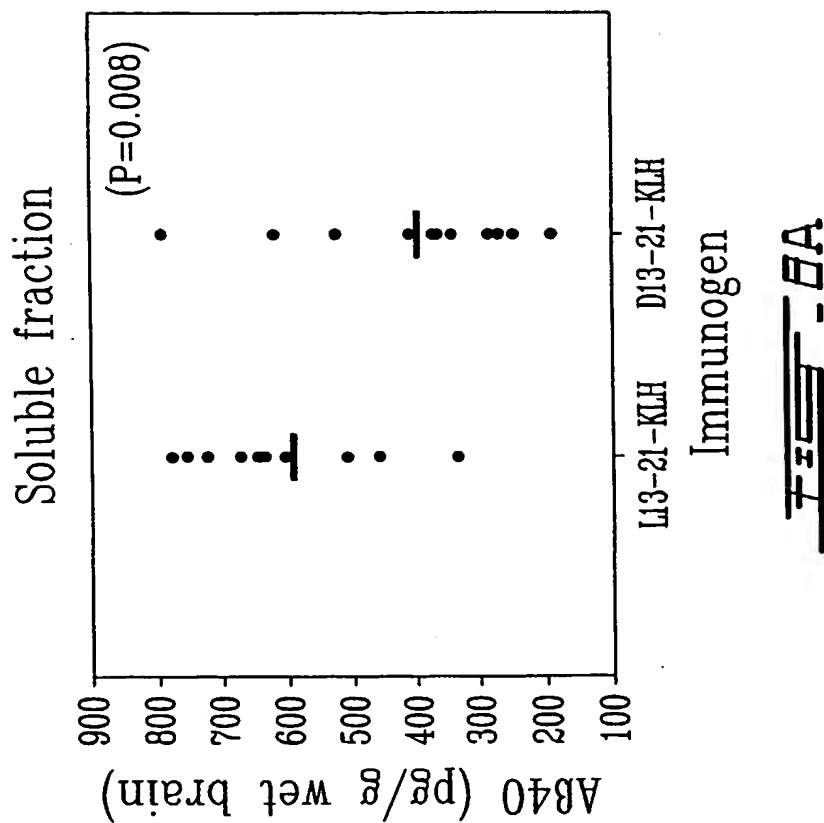
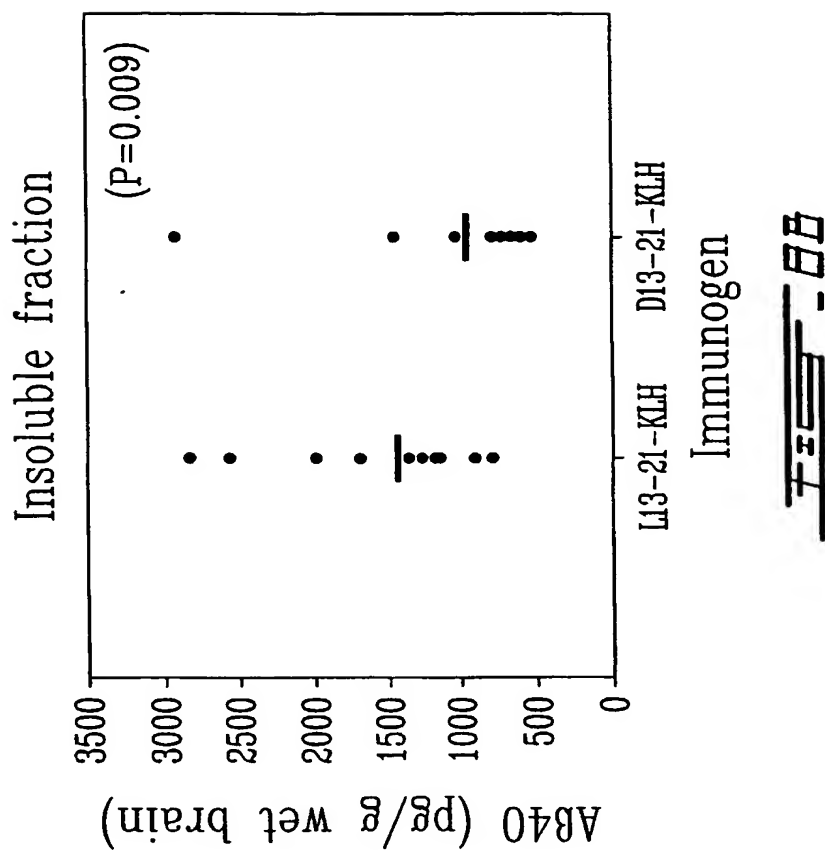
FIG. 6C

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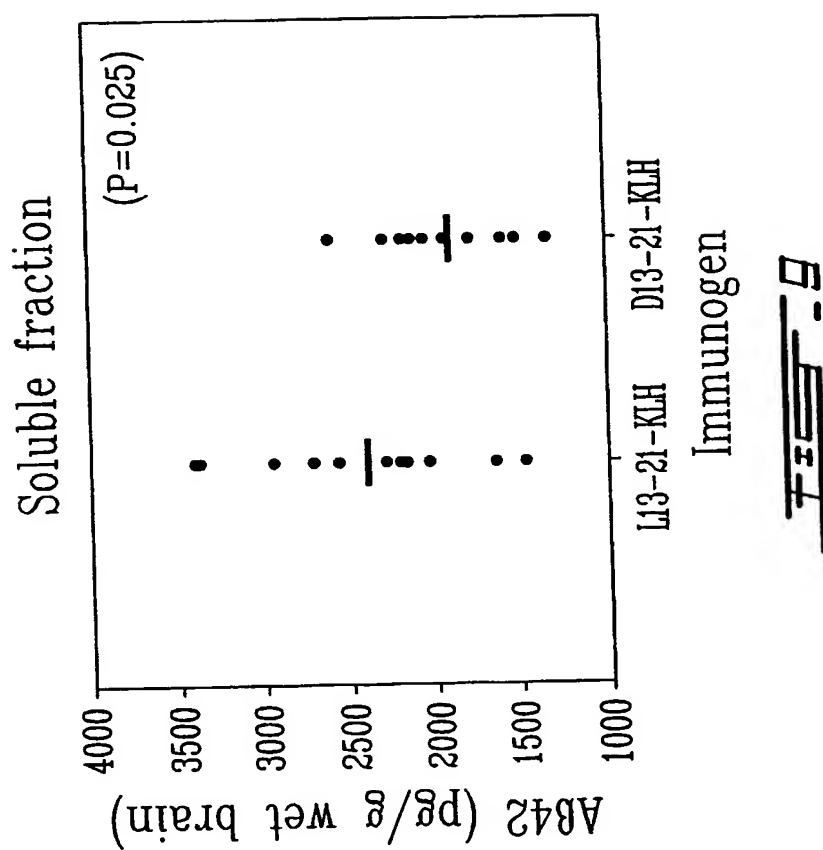


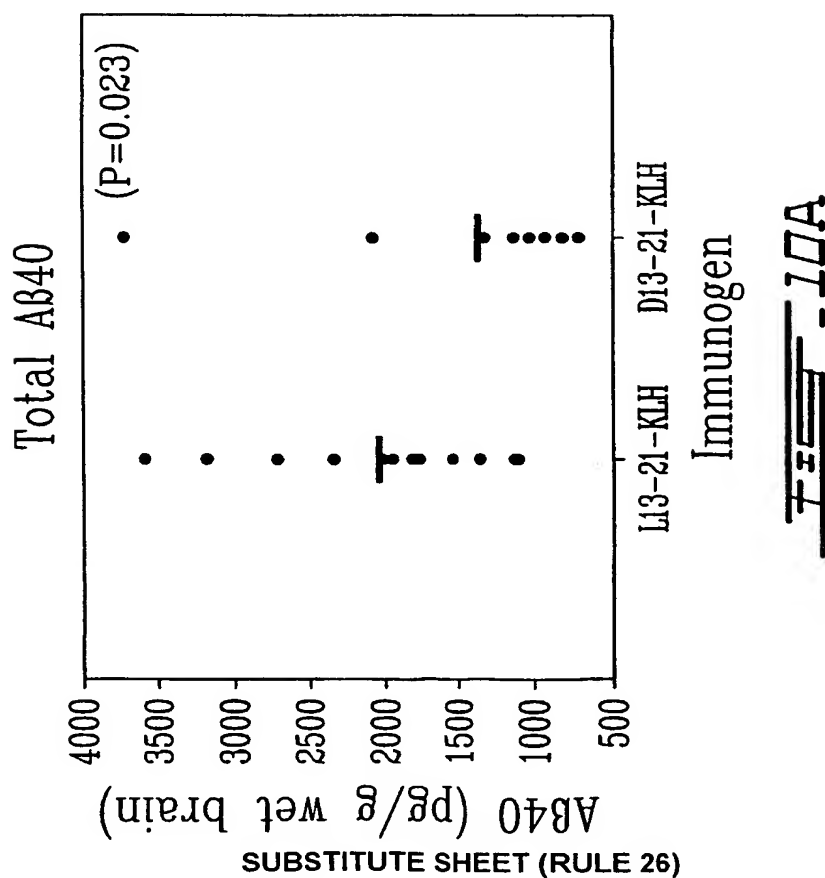
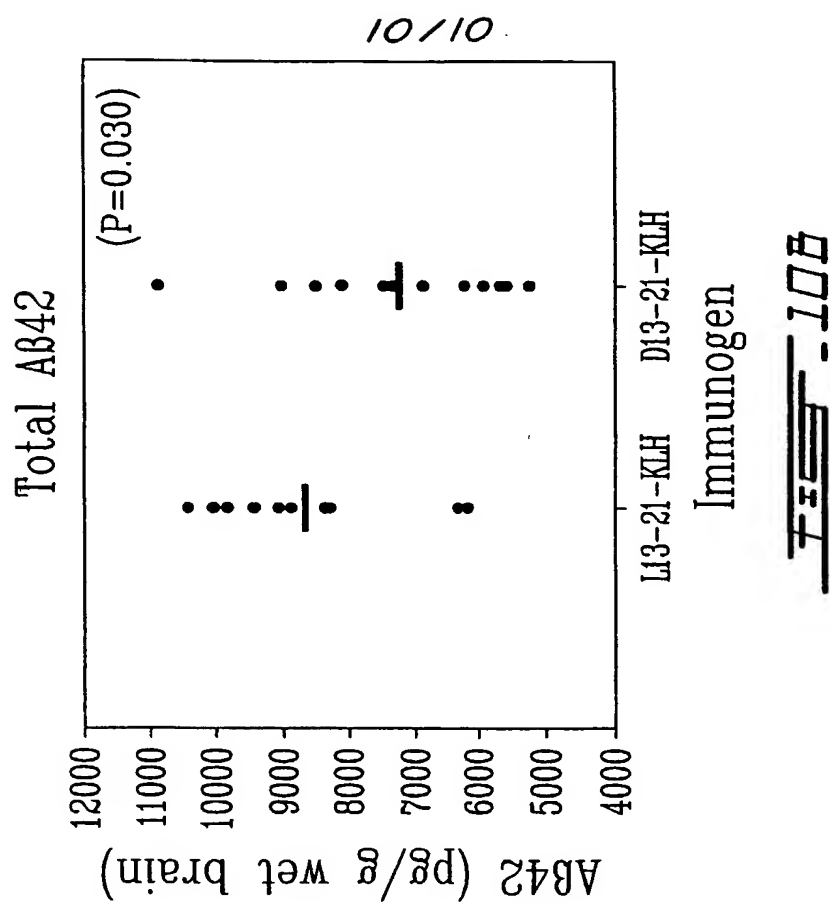


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International Bureau



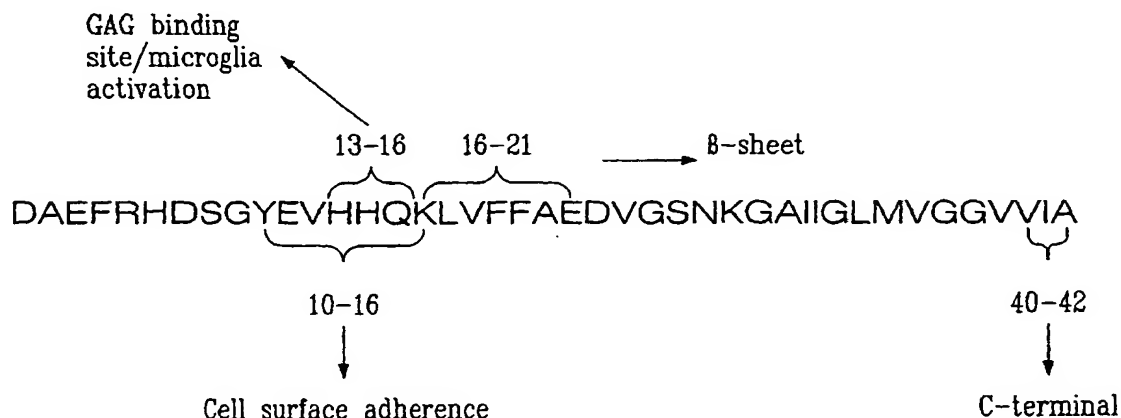
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- (30) Priority Data:  
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- (72) Inventors; and
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- (81) Designated States (national): **AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.**
- (84) Designated States (regional): **ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).**
- Published:  
— with international search report
- (88) Date of publication of the international search report:  
**10 July 2003**
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: **VACCINE FOR THE PREVENTION AND TREATMENT OF ALZHEIMER'S AND AMYLOID RELATED DISEASES**



(57) Abstract: The present invention relates to a stereochemically based "non-self" antigen vaccine for the prevention and/or treatment of Alzheimer's and other amyloid related diseases. The present invention provides a vaccine for the prevention and treatment of Alzheimer's and other amyloid related diseases, which overcomes the drawbacks associated with using naturally occurring peptides, proteins or immunogens.

## INTERNATIONAL SEARCH REPORT

onal Application No  
PCT/CA 02/00763

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 7 C07K14/47 A61K39/00 A61P25/28

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C07K A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS, WPI Data, EPO-Internal

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 00 68263 A (NEUROCHEM, INC.) 16 November 2000 (2000-11-16) the entire document, in particular pages 3, 5 and 6	1-7, 16-32
Y	WO 00 72880 A (NEURALAB LIMITED) 7 December 2000 (2000-12-07) cited in the application the entire document, in particular pages 4, 15 and 37	8-15

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

## \* Special categories of cited documents :

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

\*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

\*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

\*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

\*G\* document member of the same patent family

Date of the actual completion of the international search

12 February 2003

Date of mailing of the international search report

04/03/2003

Name and mailing address of the ISA

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Schmidt, H

## INTERNATIONAL SEARCH REPORT

International Application No  
PCT/CA 02/00763

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CRIBBS DH ET AL.: "All-D-Enantiomers of beta-Amyloid Exhibit Similar Biological Properties to All-L-beta-Amyloids" THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 272, no. 11, 14 March 1997 (1997-03-14), pages 7431-7436, XP002230811 page 7435 -----	8-15
P,X	WO 01 39796 A (NEUROCHEM INC.) 7 June 2001 (2001-06-07) the whole document -----	1-32
A	BENKIRANE N ET AL.: "Antigenicity and Immunogenicity of Modified Synthetic Peptides Containing D-Amino Acid Residues" THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 268, no. 35, 15 December 1993 (1993-12-15), pages 26279-26285, XP002164690 -----	

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/CA 02/00763

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
  
Although claims 1 to 32 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

### Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.



## INTERNATIONAL SEARCH REPORT

Information on patent family members

onal Application No

PCT/CA 02/00763

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			EP 1173480 A2	23-01-2002
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			BR 0016022 A	06-08-2002
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